Universidad Pública de Navarra
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Departamento de Ciencias del Medio Natural
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Tesis Doctoral

Pyruvate-consuming pathways as key factors in the plant physiological response after the inhibition of amino acid biosynthesis by herbicides

Memoria presentada por Dña. Miriam Gil Monreal para optar al grado de Doctor con Mención de Doctor Internacional

Pamplona-Iruñea, febrero de 2016

upna Universidad Pública de Navarra Nafarroako Unibertsitate Publikoa

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Que el trabajo titulado **"Pyruvate-consuming pathways as key factors in the plant physiological response after the inhibition of amino acid biosynthesis by herbicides"** que presenta **Dña. Miriam Gil Monreal** para optar al título de Doctor Internacional por la Universidad Pública de Navarra, ha sido desarrollado bajo su dirección en el grupo de Fisiología Vegetal y Agrobiología del Departamento de Ciencias del Medio Natural de la Universidad Pública de Navarra.

Revisado el trabajo, consideran que reúne las condiciones necesarias para su defensa, por lo que

AUTORIZAN:

La presentación de la citada Tesis Doctoral.

En Pamplona-Iruñea, febrero de 2016

Fdo: Mercedes Royuela Hernando

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Results obtained in this study have been presented in the following publications and in the following conferences:

Armendáriz O, **Gil-Monreal** M, Zulet A, Zabalza A, Royuela M (2015). Both foliar and residual applications of herbicides that inhibit amino acid biosynthesis induce alternative respiration and aerobic fermentation in pea roots. Plant Biol. doi: 10.1111/plb.12412.

Zulet A, **Gil-Monreal M,** Zabalza A, Dongen JT Van, Royuela M (2015) Fermentation and alternative oxidase contribute to the action of amino acid biosynthesis-inhibiting herbicides. *J Plant Physiol* **175**: 102–112. doi: 10.1016/j.jplph.2014.12.004.

Gil-Monreal M, Licausi, F.; Giuntoli, B.; Fernández-Escalada, M, Zabalza A, Royuela M (2015). Transcriptional regulation of ethanol fermentation in the toxicity of amino acid biosynthesis inhibiting herbicides. XXI Reunión de la Sociedad Española de Fisiología Vegetal - XIV Congreso Hispano-luso de Fisiología Vegetal. *Current Research in Plant Physiology*. ISBN 978-84-606-8883-9.

Gil-Monreal, M., Missihoun, T.D., Bartels, D., Zabalza, A., Royuela, M. (2014) Aldehyde dehydrogenases in the mode of action of amino acid biosynthesis-inhibiting herbicides. FESPB/EPSO 2014 Congress.

Royuela, M., Zulet, A., **Gil-Monreal, M.**, Armendáriz, O., Zabalza, A. (2013) Comparación de la aplicación foliar y radical de herbicidas inhibidores de la biosíntesis de aminoácidos. XIV Congreso Sociedad Española de Malherbología. ISBN: 978-84-9048-131-8.

Gil-Monreal M, Zulet A, Armendáriz O, Zabalza A, Royuela M (2011) Función de la fermentación en la respuesta de las plantas a los herbicidas inhibidores de la biosíntesis de aminoácidos ramificados. XIX Reunión Sociedad Española de Fisiología Vegetal- XII Congreso Hispano-Luso de Fisiología Vegetal. ISBN: 978-84-8021-805-4.

This work was financially supported by a grant from the Ministerio Español de Ciencia y Tecnología (AGL-2010-18621-AGR) and a grant from the Ministerio Español de Economía y Competitividad (AGL-2013-40567-R).

Miriam Gil Monreal has been holder of a fellowship from the Public University of Navarra and she has received two mobility grants from the same institution

A la abuela Vale

AGRADECIMIENTOS

En primer lugar, me gustaría dar las gracias a mis directoras de tesis, Mercedes y Ana, por haberme dado la oportunidad de realizar esta tesis doctoral en su grupo de investigación. Eskerrik asko Mercedes por tu apoyo, tus buenas ideas, tu capacidad de dar claridad a los resultados y hacer fácil lo difícil. Eskerrik asko Ana por tu disponibilidad y cercanía, por tu apoyo, estar siempre dispuesta a escucharme y tirar de mí, no sin sacarme antes una sonrisa.

I would like to express my sincere gratitude to Prof. Dr. Dorothea Bartels for giving me the opportunity to work on your lab. I would especially like to thank Dr. Tagnon for your help during and after my stay and for your positive energy. I would also like to thank all the members of the big lab, especially to Paco, Saeedeh, Valentino, Dinakar, Naim, and Karolina, for doing my stay easier.

I would also like to thank Prof. Dr. Peter Doermann for opening the door of your lab. Thanks to Helga for your hard work, your clarity and your closeness. Vielen dank!

Thank you Francesco for receiving me with open arms for my stay in Pisa. Thanks also to Bea, for all your help, and to all the people from the PlantLab: Pierdominico Perata, Antonietta, Giacomo, Chiara, Elena, Liem, Silvia, Moon, Claudia, Dubravka; and of course, to Hans, Francesco G. and Maria. Hope to see you again!

También quisiera expresar mi agradecimiento a Lucía Ramírez y Gerardo Pisabarro, catedráticos del grupo de Genética del departamento de Producción Agraria de la UPNA, por abrirme las puertas de su laboratorio y dejarme utilizar todo cuanto fuera necesario. Gracias también a Manu, Raúl, Aless y Alejandra por esos ratikos en los que no todos fueron ciencia.

Gracias a todos mis compañeros de CCMN. A los doctores Pedro, Esther, Cesar, Iker, Berta y Morán. A Gustavo, por toda tu ayuda en el laboratorio, y a Susana, como voy a echar de menos esas comidas! Gracias también a todos los miembros que habéis formado parte de secretaría: Encarna, Alfredo y Teresa.

Gracias a mis compañeros del grupo herbicidas. A Amaia, qué te voy a decir ya que no sepas, Gracias guapa! Y a Oskar, por esos momentos compartidos dentro y fuera del laboratorio, aún nos queda Jurramendi! Ay cuánto me hubiera gustado que estuvierais aquí en esta última etapa! Gracias también a mis compañeros de la recta final, Ainhoa y Manu, ánimo con vuestras tesis, que no se os resistan las Amaranthus! A Micaela, por tu alegría y tu buena actitud en el trabajo, me ha encantado fermentar contigo. Y a Diana, por tu energía y disponibilidad en el trabajo.

Gracias a todos l@s becari@s del departamento. A mi txantxita Amaia, por estar ahí, por tu apoyo y por todos los buenos ratos. Ánimo, que ya no queda nada! Eskerrik asko Joseba hor egoteagatik, zure laguntzarengatik eta momentu guztiengatik. A Esti por tus sabios consejos y por estar siempre dispuesta a responder todas mis mil dudas moleculares. A mi txantxita Erena, mila esker zure gomendioengatik eta solasaldi

luzeengatik. A Irantzu y Maitane, mis compañeras de fatiga, por los buenos momentos disfrutados.

Y gracias a tod@s los demás becari@s que habéis pasado por CCMN: Maribel, Idoia, Inma, Janaina, Cristina, Olaya, Ivan, Xabi, Ester, Maren, Karla, Verónica y Libertad (espero no dejarme a nadie).

Gracias a los compañeros del Idab, donde tuve mi primer contacto con el departamento, a Bea, Laura, Aaron, Edurne, Esti U. y Rakel.

Al "Komando Pilinguis" por esas conversaciones filosóficas en el Toki que me han servido para desconectar. A mi "segunda kuadrila" por los buenos ratos pasados que me han hecho salir de esta burbuja.

Eskerrik asko nire biolololokei, Katerin eta Kristina, zareten bezalakoak zaretelako. Gracias a las "sexys y biólogas" Palo, Ainhoa, Guti y Amaia.

Y por supuesto, eskerrik asko Aita y Ama, muchas gracias por confiar en mí, por haberme permitido llegar hasta aquí, por vuestro apoyo y cariño. Eskerrik asko nire anai-arrebei, Eneko eta Oihane, zuen laguntza eta ardurarengatik.

Gracias a toda mi familia, que os habéis preocupado por saber cómo me iba y qué tal estaba y por el cariño recibido durante este tiempo. Eskerrik asko denoi.

Eta azkenik, Haritz: eskerrik asko zure laguntzarengatik, beti ni entzuteko prest egoteagatik eta egunero zaintzeagatik. Eskerrik asko hor egoteagatik!

ABBREVIATIONS

AAA	Aromatic amino acid
OD	Optical density
4-MU	4-methylumbelliferone
ABIH	Amino acid biosynthesis inhibiting herbicides
ACBP	Acyl-CoA-binding proteins
ACC2	Acetyl-CoA carboxylase
ACP	Acyl carrier protein
ACS	Acetyl-CoA synthetase
ADH	Alcohol dehydrogenase
AHAS	Acetohydroxyacid synthase
ALDH	Aldehyde dehydrogenase
AMADH	Aminoaldehyde dehydrogenase
ANOVA	Analysis of variance
BCAA	Branched-chain amino acid
BHT	Butylated hydroxytoluene
BSA	Bovine serum albumin
C	Control
Col-0	Columbia-0
DAHPS	3-deoxy-d-arabino-heptulosonate-7-phosphate synthase
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
Dnase	Deoxyribonuclease
DTT	Dithiothreitol
DW	Dry weight
E1	Ub-activating enzyme
E2	Ub-conjugating enzyme
E3	Ub ligase
EDTA	Ethylenediaminetetraacetic acid
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
ERF	Ethylene response factors
FAS	Fatty acid synthase
FAT	Fatty acyl thioesterases
FI	Fluorescence intensity
FW	Fresh weight
GABA	γ-aminobutyric acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GLP	Glyphosate
GS	Glutamine synthetase

GSH	Reduced glutathione
GSSG	Oxidized glutathione
GUS	β-glucuronidase
HB	Hemoglobin
HRAC	Herbicide resistance action committee
HRP	Horseradish peroxidase
IBAA	Inhibidores de la biosíntesis de aminoácidos aromáticos
IBAR	Inhibidores de la biosíntesis de aminoácidos ramificados
IMX	Imazamox
KARI	Ketolacid Reducto-isomerase
KAS	3-ketoacyl-ACP synthases
LDH	Lactate dehydrogenase
LOW-OX	Low-oxygen
MAP	Methionine amino peptidase
MCMT	Malonyl-coa:ACP-malonyltransferase
MDA	Malondialdehyde
MOPS	3-(N-morpholino)propanesulfonic acid
MS	Murashige and Skoog
MTAB	Myristyltrimethylammonium bromide
MUG	4-methylumbelliferyl-beta-D-glucuronide
NERP	N-end rule pathtway
NO	Nitric oxide
NTSR	Non-target-site resistance
PAGE	Polyacrylamide gel electrophoresis
PCO	Plant cysteine oxidase
PDC	Pyruvate decarboxylase
PDH	Pyruvate dehydrogenase
PEP	Phosphoenolpyruvate
PRT	Proteolysis
PRT	Proteolysis
PVDF	Polyvinylidene fluoride
qPCR	Quantitative real time polymerase chain reaction
RNA	Ribonucleic acid
RNAse	Ribonuclease
ROS	Reactive oxygen species
RS	Reagent solution
RT-PCR	Reverse transcription polymerase chain reaction
R-transferases	Arginyl-tRNA-transferases
SAD	Stromal Δ^9 stearoyl-ACP desaturase
SDS	Sodium dodecacyl sulphate
SE	Standard error

TBA	Thiobarbituric acid
TCA	Tricarboxylic acid
TF	Transcription factor
TPP	Thiamine pyrophosphate
Tris	Tris(hydroxymethyl)aminomethane
TSR	Target-site resistance
TTBS	Tween tris buffer saline
Ub	Ubiquitin
UPS	Ubiquitin proteasome system
wt	Wild-type
X-Gluc	5-bromo-4-chloro-3-indolyl-β-D-glucuronide

SUMMARY

Herbicide application makes major contributions to global food production by easily removing weeds, however, herbicide efficacy is now compromised by the rapid evolution of resistant weeds. Knowing more about the physiological side effects triggered in plants after herbicide application would help to elucidate why plants die as a consequence of herbicide treatment and would help in the discovery of new herbicides with new sites of action that will reduce the selection pressure for resistant weeds.

The general aim of the present thesis is to gain further insights in the common physiological effects provoked by branched-chain amino acid (BCAA) and aromatic amino acid (AAA) biosynthesis inhibiting herbicides. Although they target different enzymes, several common physiological effects have been described for both types of herbicides, suggesting that they kill plants by similar mechanisms.

One common physiological effect triggered by the application of amino acid biosynthesis inhibiting herbicides (ABIHs) is the induction of the aerobic ethanol fermentation. The role of this pathway in the response of the plants to the application of BCAA and AAA biosynthesis inhibiting herbicides was investigated and it was detected to be species-dependent: while in pea plants the ethanol fermentation pathway reduces the effects triggered after the application of the herbicides, in *Arabidopsis thaliana* plants induces the effects triggered, contributing to the toxicity provoked by the herbicides.

The induction of the ethanol fermentation is transcriptionally regulated in plants upon treatment with ABIHs, but this regulation differs from the regulation of the ethanol fermentation described in plants exposed to low-oxygen conditions, because no implication of the transcription factor RAP2.12 was found. The role of pyruvate, the substrate of the ethanol fermentation pathway, in the regulation of ethanol fermentation was also investigated. The results showed that pyruvate participates in the regulation of the ethanol fermentation in plants treated with ABIHs, but its role cannot be only explained by a mimicked effect or a higher substrate availability.

The possible participation of selected aldehyde dehydrogenases in the response of the plants to ABIH application was evaluated and it was found that the upregulation of the *ALDH7B4* is another common effect triggered as a consequence of ABIH application. The induction of *ALDH7B4* seems to alleviate the stress provoked by the herbicides on roots carbon metabolism and, contrary to what it has been described for other stresses, it could not be related to an alleviation of an oxidative stress. Finally, it was observed that the PDH-bypass (another pyruvate-consuming pathway) is also activated in plants treated with ABIHs, but it could not be elucidated if this pathway contributes to the *de novo* fatty acid biosynthesis in ABIH-treated plants, or by contrast it has a different role.

To sum up, this study provides new insights in the induction of fermentation triggered after the application of BCAA and AAA biosynthesis inhibiting herbicides and reveals new common physiological effects provoked by these types of herbicides, supporting the hypothesis that both types of herbicides provoke plant death by a similar mechanism.

RESUMEN

Actualmente, el uso de herbicidas contribuye a mantener una alta producción en la agricultura, ya que permiten controlar las malas hierbas de una manera muy eficaz. Sin embargo, el uso de estos compuestos se está viendo limitado por el rápido desarrollo de malas hierbas resistentes a los herbicidas. Conocer con exactitud los efectos que estos compuestos provocan en las plantas, ayudaría a comprender qué es lo que provoca la muerte de las plantas tratadas, y en el desarrollo de nuevos herbicidas que inhiban nuevos procesos y alivien la presión de selección que conduce a la selección de poblaciones resistentes.

El objetivo general de este trabajo es profundizar en el conocimiento de los procesos fisiológicos provocados por los herbicidas inhibidores de la biosíntesis de aminoácidos ramificados (IBAR) y aromáticos (IBAA). Aunque estos herbicidas inhiben dianas diferentes, se ha descrito que provocan efectos fisiológicos comunes en las plantas tratadas, sugiriendo que pueden provocar la muerte de las plantas por un mecanismo similar.

Uno de los efectos fisiológicos comunes provocados en las plantas por los herbicidas IBAR y los IBAA, es la inducción de la ruta de fermentación etanólica, a pesar de que las plantas crecen en condiciones aeróbicas. En este trabajo se ha profundizado en el papel de la fermentación etanólica en la respuesta de las plantas a la aplicación de los herbicidas IBAR e IBAA. Los resultados demostraron que la inducción de la fermentación etanólica cumple papeles diferentes según la especie estudiada. En las plantas de guisante, la fermentación alivia los efectos fisiológicos más característicos provocados por los herbicidas. Por el contrario, en plantas de *Arabidopsis thaliana* parece que la fermentación etanólica contribuye a la toxicidad provocada por los herbicidas.

Por otro lado, también se estudió la regulación de dicha inducción a nivel transcripcional. Los resultados mostraron que esta ruta está regulada a nivel transcripcional tras la aplicación de los herbicidas, pero que dicha regulación es diferente a la regulación descrita en plantas crecidas en condiciones de falta de oxígeno. Al contrario de lo que ocurre en condiciones limitantes de oxígeno, el factor de transcripción RAP2.12 no participa en la regulación transcripcional de

la ruta de fermentación etanólica en las plantas tratadas con herbicidas IBAR e IBAA. Asimismo, se estudió el papel del piruvato, sustrato de la fermentación etanólica, en la regulación de dicha ruta. Se comprobó que este metabolito participa en la regulación de la fermentación etanólica en las plantas tratadas con los herbicidas IBAR e IBAA, con un papel más complejo que el aumento en la disponibilidad de sustrato.

Adicionalmente, se analizó el papel de diferentes aldehído deshidrogenasas en los efectos provocados por los herbicidas IBAR e IBAA. Los resultados demostraron que como consecuencia de la aplicación de ambos tipos de herbicidas, se induce la expresión del gen *ALDH7B4*, un efecto común para ambos grupos de herbicidas hasta ahora no descrito. En cuanto al papel de la inducción del gen *ALDH7B4*, se observó que podría inducirse para aliviar los efectos de los herbicidas en el metabolismo del carbono a nivel de raíz, y al contrario de lo observado en otros estreses, no parece estar relacionado con el alivio de un estrés oxidativo. Finalmente, también se comprobó que ambos tipos de herbicidas provocan la inducción del "PDH-bypass" (otra ruta cuyo sustrato es el piruvato). Sin embargo, no se pudo esclarecer si dicha ruta contribuye a la síntesis de ácidos grasos en las plantas tratadas con herbicidas IBAR e IBAA, o si por el contrario, cumple otro papel.

En resumen, este trabajo proporciona nuevos detalles en cuanto a los efectos fisiológicos comunes provocados en las plantas por los herbicidas IBAR e IBAA, y revela nuevos efectos comunes para ambos tipos de herbicidas. Por tanto, estos resultados apoyan la hipótesis de que ambos grupos de herbicidas provocan la muerte de las plantas por un mecanismo similar a pesar de que inhiben rutas diferentes.

LABURPENA

Gaur egun, nekazaritzan ekoizpen maila altuak mantentzeko herbizidak oso lagungarriak dira, hauek oso eraginkorrak baitira belar txarrak suntsitzeko. Baina, produktu hauen erabilera mugatzen ari da, hauekiko erresistenteak diren belar txarren agerpena dela eta. Herbizidek landareetan gauzatzen dituzten eragin fisiologikoak ezagutuko bagenitu, konposatu hauek landareak nola hiltzen dituzten ulertuko genuke, eta informazio horrek selekzio presio txikiagoa duten herbizida berrien diseinuan lagunduko liguke.

Lan honen helburua, hain zuzen, aminoazido adarkatuen edo aromatikoen biosintesia inhibitzen duten herbizidek gauzatzen dituzten eragin fisiologikoen ezagutzan sakontzea da. Bi herbizida mota hauek prozesu desberdinak blokeatu arren, landareetan eragin fisiologiko komun asko eragiten dituzte, beraz, hauen heriotza mekanismo berdintsu baten bitartez eragiten dutela pentsa daiteke.

Aminoazido adarkatu eta aromatikoen biosintesia inhibitzen duten herbizidek landareetan gauzatzen dituzten eragin fisiologiko berdinen artean, hartzidura alkoholikoaren bizkortzea aurkitzen da. Lan honetan, herbiziden erabileraren ondorioz landareetan hartzidurak duen garrantzian sakondu da. Hartzidurak landare espeziearen arabera paper desberdina duela iradokitzen dute emaitzek. Ilarretan, hartzidurak herbiziden eragina txikiagotzen laguntzen duela dirudi, aldiz *Arabidopsis thaliana*-n, herbiziden eragina areagotzen duela dirudi.

Bestalde, hartziduraren erregulazioa transkripzio mailan aztertu da herbizidekin tratatutako landareetan. Emaitzek hartzidura transkripzio mailan erregulatua dagoela adierazten dute, baina, erregulazio honetan RAP2.12 transkipzio faktoreak parte hartzen ez duela erakusten dute. RAP2.12 transkripzio faktoreak oxigeno faltaren ondorioz hartziduraren bizkortzea erregulatzen du, beraz, hartziduraren erregulazioa desberdina da herbiziden eraginez edo oxigeno faltaren ondorioz sustatzen bada. Gainera, pirubatoak (hartziduraren sustratoa) hartziduraren erregulazioan duen papera aztertu da eta konposatu honek prozesu honen erregulazioan parte hartzen duela ikusi da

herbizidekin tratatutako landareetan eta pirubatoaren papera soilik bere erabilgarritasunaren igoerarekin lotuta ez dagoela frogatu da.

Gainera, herbizidek gauzatutako eragin fisiologikoetan aldehido deshidrogenasek parte hartzen duten ala ez ikertu da. *ALDH7B4* genearen espresioaren areagotzea herbizida mota hauen beste eragin fisiologiko komun bat dela egiaztatu da. Gene honen espresioaren igoerak, herbizidek sustraien karbonoaren metabolismoan duten eragina ahultzen laguntzen duela dirudi. Beste estres mota batzuetan ikusi denarekin kontra eginez, gene honek herbizidekin tratatutako landareetan estres oxidatibo batekin harremanik ez duela dirudi. Azkenik, aminoazido adarkatu edo aromatikoen biosintesia inhibitzen duten herbiziden ondorioz, "PDH-bypass"-a ere aktibatzen da. Ibilbide honek, hartzidurak ez bezala, pirubatoa ere kontsumitzen du. Hala ere, ezin izan da ibilbide honek gantz-azidoen sintesian laguntzen duen ala beste funtzio batzuetan laguntzen duen egiaztatu.

Jasotako emaitzek, aminoazido adarkatuen edo aromatikoen biosintesia eragozten duten herbizidek, prozesu desberdinak blokeatu arren, landareetan eragin fisiologiko berdin asko gauzatzen dituztela iradokitzen digute. Lan honetan bi eragin fisiologiko komun berri aurkitu dira: *ALDH7B4* genearen espresioaren handiagotzea eta "PDH-bypass"-aren aktibazioa. Beraz, bi herbizida mota hauek landareak mekanismo berdintsu baten bitartez hiltzen dituztela ondorioztatu dezakegu.

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- GENERAL INTRODUCTION -

A. HERBICIDES: GENERAL OVERVIEW AND CURRENT SITUATION

Successful crop production depends to a great extent on a proper weed management, since the presence of weeds in crops reduces yields (Cobb and Reade, 2010). Weeds compete with the crop for the available resources (e.g. water, nutrients, light and space), can host pests that can possibly affect the crop, interfere with the recollection and, thus, increment crop production costs.

There are several methods to prevent weed growth, among which herbicide application, in coordination with other agronomic and mechanic methods, is the most extensively used one. Herbicides are chemical products that inhibit the growth of unwanted plants. They target specific plant enzymes that take part in essential metabolic pathways for the plant. This first biochemical or metabolic process that is specifically affected by each herbicide is often known as the site of action of the herbicides.

The efficacy of the herbicides is stated by their chemical properties since they determine the ability to enter the plant, be translocated, and reach the target-site at a lethal dose. Once the herbicide reaches the target enzyme, many physiological effects are triggered before plant death occurs. Although the site of action has been well established for most of the commercialized herbicides, the contribution of the physiological side effects to the plant death is not fully understood, which means that the exact reason why plant dies after herbicide application is not completely known.

In general, there are three reasons that lead to plant death once the target-site is affected. First, as a consequence of the metabolic pathway inhibition a deficiency of the end products can lead to plant death. Second, an accumulation of the substrates of the inhibited enzyme can be associated with the plant death. And third, several side reactions can be triggered after the target inhibition that can lead to the plant death. Knowledge of the mechanisms involved in plant death in response to herbicides could provide a better understanding of the plant metabolism and could be useful in the design of new herbicides.

Currently, there are 311 active ingredients available for the different commercial herbicide formulations (Heap, 2016) from which 120 are permitted to be used for weed control in Spain (Regulation (EC) Nº 1107/2009). Herbicides can be classified attending to different parameters, such as, the method of application, the timing of application and the site of action.

Attending to their site of action, the Herbicide Resistance Action Committee (HRAC) differentiates 17 groups of herbicides, which are named with the letters of the alphabet (Figure A.1) (HRAC, 2016).

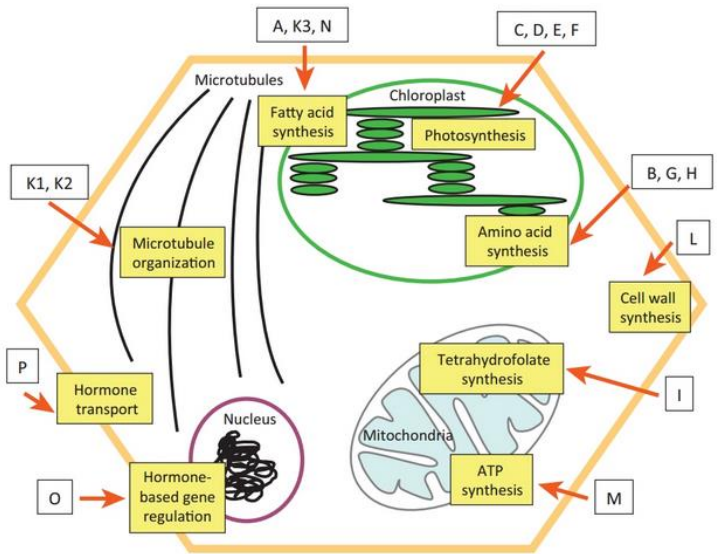


Figure A.1. Cellular targets of herbicide action and herbicide classification by site of action according to the Herbicide Resistance Action Committee (HRAC) (Délye et al., 2013).

In Europe, the use of herbicides is regulated by The Regulation (EC) №1107/2009 and the Directive 2009/128/EC of the European Parliament and of the Council, which regulate the commercialization and state a sustainable use of pesticides. As the concern about the environmental risks that the application of pesticides involves is increasing, the legislation that regulates the use of these chemical compounds is becoming stricter, and consequently, the introduction of new herbicides is becoming much more expensive and complicated.

Moreover, the demand for new herbicides has decreased as a result of the introduction of the herbicide resistant crops (Duke, 2012). Consequently, no new herbicide site of action has been introduced for about 20 years (Duke, 2012).

B. RESISTANCES TO HERBICIDES

After the commercialization of the first herbicide in the 1940s, the use of these compounds rapidly increased. The herbicides reduce crop production costs since they eliminate weeds more rapidly and in a more economically efficient way than the mechanic or manual methods. However, the use of herbicides also has some drawbacks. The herbicides can persist on soils, contaminate groundwaters and reach non-target organisms (e.g. soil microbes, animals and non-target plants) (Cheng, 1990). Moreover, the use of herbicides increases the selection pressure for resistant weeds.

Currently, 249 species (144 dicots and 105 monocots) have been found to present biotypes that are resistant to one or more group of herbicides (classified by site of action) (Heap, 2016). Figure B.1 shows the number of species showing biotypes resistant to the different groups of herbicides (according to HRAC classification) along the time. Only the herbicide groups that present the highest number of resistant biotypes are shown.

Many factors influence the development of resistant weeds, including the biology of the weed species, the characteristics of the applied herbicide, the selection pressure, the existing genetic diversity and the nature of the target-site (Powles and Yu, 2010). The evolution of resistant weeds can be very rapid and appear within few years after the introduction of the herbicide they are resistant to (e.g. 3-4 years for acetohydroxyacid synthase (AHAS) inhibitors) or it can be slower and take decades (e.g. around 20 years for glyphosate (GLP)).

The introduction of herbicide-resistant crops has made the situation worse because it has lead the farmers rely on the application of a single herbicide, and as a consequence, the selection pressure for resistant weeds has increased. The

efficacy of the existing herbicides is decreasing due to the rapid development of resistant weeds. There is a demand for new herbicides with new sites of action, which could be based on the physiological effects caused by the currently existing herbicides. Moreover, the need for new herbicides is supported by the fact that the number of existing herbicides in agriculture has declined, because some of them have been prohibited.

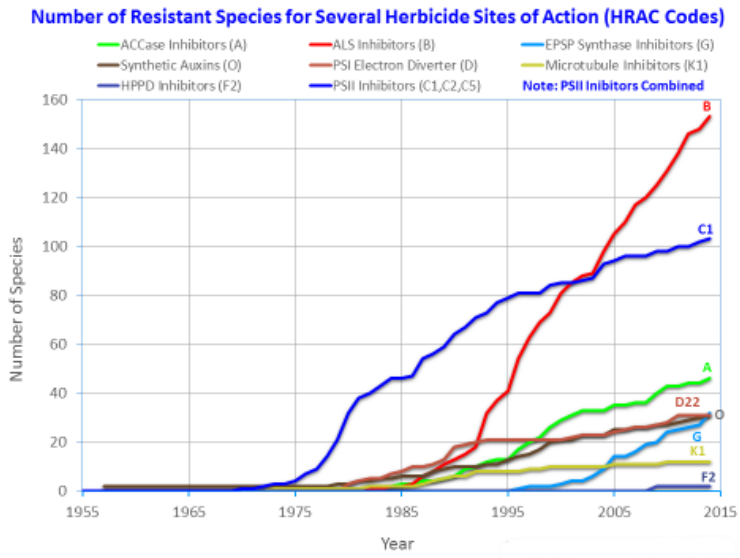


Figure B.1. The chronological increase in the number of resistant biotypes to different herbicide groups classified by their site of action. The letters refer to the Herbicide Resistance Action Committee (HRAC) code to identify herbicide site of action. Note that only the eight groups showing the highest number of resistant biotypes are shown (Heap, 2016).

There are two mechanisms by which weeds can be resistant to herbicides, known as target-site resistance (TSR) and non-target-site resistance (NTSR) (Powles and Yu, 2010). Evolved TSR occurs when the applied herbicide reaches the target-site, but its effect is limited because of changes in the target-site. On the other hand, evolved NTSR exists when the amount of herbicide that reaches the target-site is minimized by mechanisms present in the plant.

B. 1. Target-Site Herbicide Resistance

There are two mechanisms by which TSR can evolve. On the one hand, mutations on the gene coding for the target protein that confer changes in the amino acid sequence of the target enzyme, can prevent herbicide binding and limit its efficacy. On the other hand, overexpression of the target enzyme, by gene amplification or gene promoter changes, can also reduce the herbicide efficacy. The main problem of the TSR is that, often, weeds showing TSR are resistant not only to one single herbicide, but to different herbicides with the same target-site.

B. 2. Non-Target-Site Herbicide Resistance

When the amount of herbicide that reaches the target enzyme is limited to a nonlethal dose by one or more mechanisms present in the plant, NTSR is evolved. There are different mechanisms that confer NTSR, such as, the decrease in the herbicide penetration, the limitation of the herbicide translocation, the sequestration of the herbicide into vacuoles, and the increase in the herbicide detoxification pathways. Herbicides can be detoxified by cytochrome P450 monooxygenases, a threatening detoxification pathway since these enzymes can metabolize different herbicides with different site of actions conferring cross-resistance to the resistant plant (Powles and Yu, 2010). Glutathione S-Transferases has also been described to detoxify herbicides by conjugation with glutathione. Glutathione-conjugated herbicides can be then accumulated in the vacuole (Martinoia et al., 1993) or be eliminated through the root tips (Schröder et al., 2007). Unlike the TSR, the NTSR mechanisms can sometimes be more universal and confer resistance to several herbicides with different target-sites, leading to cross-resistance.

C. AMINO ACID BIOSYNTHESIS-INHIBITING HERBICIDES (ABIHs)

Among the existing herbicides, there are three groups that inhibit the biosynthesis of amino acids (named B, G and H, according to HRAC classification) and, interestingly, they are the groups to whom the most worldwide used herbicides belong. Since these herbicides inhibit pathways only

present in plants and microorganisms, they tend to be less toxic for animals, including mammals, than other herbicides (Reade and Cobb, 2002).

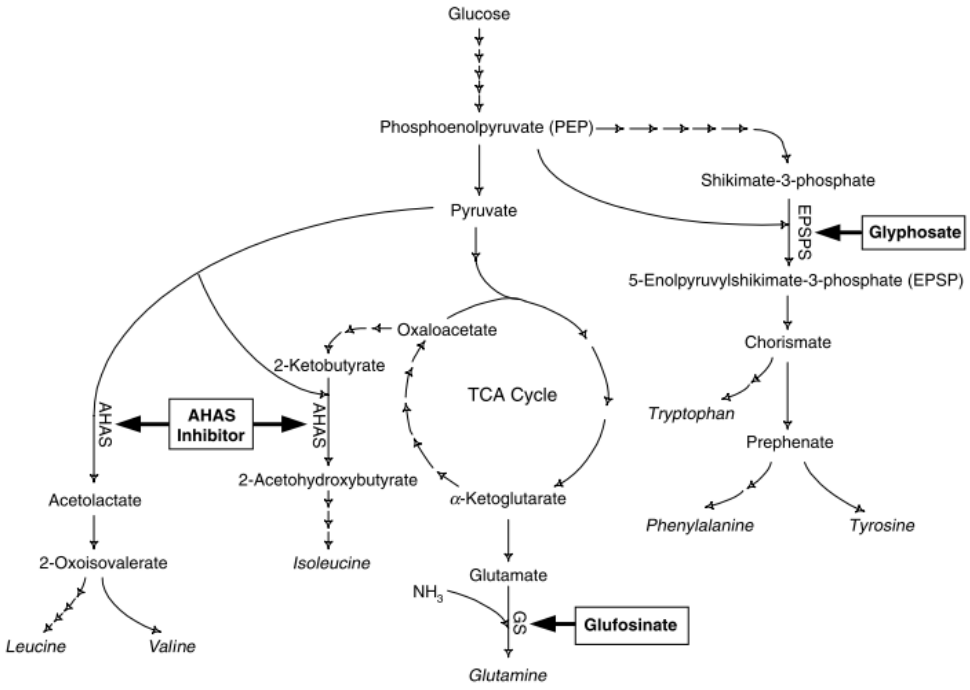


Figure C.1. Branched-chain amino acid, aromatic amino acid and glutamine biosynthesis pathways in plants and the specific inhibitors of each pathway. Acetohydroxyacid synthase (AHAS) inhibitors inhibit AHAS in the branched-chain amino acid pathway, glufosinate inhibits glutamine synthase (GS) in the glutamine biosynthesis pathway and glyphosate inhibits 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) in the aromatic amino acid biosynthesis pathway (Tan et al., 2006).

Three are the targets for commercially available ABIHs (Figure C.1): herbicides included in the group B (HRAC classification) inhibit the enzyme AHAS (also referred as to acetolactate synthase) (EC 2.2.1.6) in the branched-chain amino acid (BCAA) biosynthesis; herbicides belonging to the group G (HRAC classification) inhibit the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (EC 2.5.1.19) in the aromatic amino acid (AAA) biosynthesis; and herbicides included in the group H (HRAC classification) inhibit the enzyme glutamine synthetase (GS) (EC 6.3.1.2) in the glutamine synthesis

(Duke, 1990). The study presented here focuses on the herbicides from Group B and Group G.

C. 1. Branched-chain amino acid biosynthesis-inhibiting herbicides (Group B)

Bacteria, archaea, fungi and plants are able to synthesize BCAAs (valine, leucine and isoleucine), instead, animals, including humans, have to take up these amino acids with their diets. Val and Ile synthesis occurs in parallel with a single set of four enzymes (AHAS; Ketolacid Reducto-isomerase (KARI) (EC 1.1.1.86); Dihydroxyacid Dehydratase (EC 4.2.1.9) and Branched-Chain Aminotransferase (EC 2.6.1.42)) that utilize different substrates (Figure C.2) (Singh, 1999). Nevertheless, there is a previous reaction in the Ile biosynthesis before the common route starts that is catalysed by Threonine deaminase (EC 4.2.1.19) (Figure C.2). In contrast, Leu biosynthesis starts from 3-Methyl-2-oxobutanoate which is the last intermediate that is transaminated to form Val (Figure C.2).

AHAS is the first common enzyme in the BCAA biosynthesis pathway (Figure C.1 and C.2). Although it is a nuclear encoded enzyme, its activity is localized in the plastids, where the amino acid biosynthesis takes place. On the one branch, AHAS catalyses the condensation of pyruvate and 2-Oxobutanoate to form CO_2 and 2-Aceto-2-hydroxybutanoate, in the Ile biosynthesis pathway (Figure C.2) (Singh and Shaner, 1995). On the other branch, AHAS also catalyses the condensation of two pyruvate molecules to produce CO_2 and 2-Acetolactate in the Val and Leu biosynthesis (Figure C.2) (Singh and Shaner, 1995)

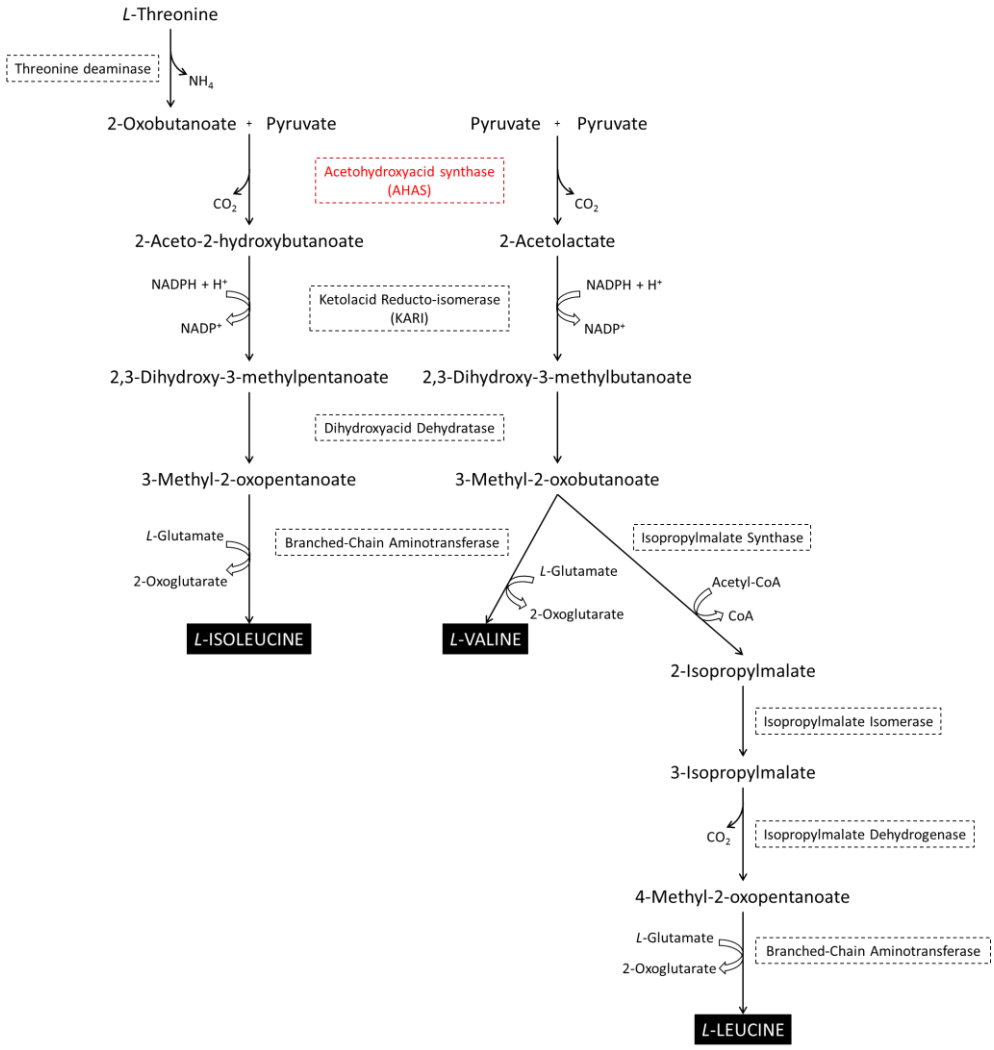


Figure C.2. Branched-chain amino acid (BCAA) biosynthesis pathway in plants. Acetohydroxyacid synthase (AHAS) (represented in red) is the first common enzyme in the BCAA pathway and it is specifically inhibited by the commercialized BCAA biosynthesis-inhibiting herbicides.

Although all the commercialized BCAA biosynthesis-inhibiting herbicides are AHAS inhibitors, efforts have been made to develop herbicides that inhibit other enzymes in the BCAA biosynthesis pathway. Inhibitors of the KARI have been found. This enzyme catalyses the reductive isomerization of 2-Acetolactate to 2,3-Dihydroxy-3-methylbutanoate (in the Leu and Val synthesis) or the

conversion of 2-Aceto-2-hydroxybutanoate to 2,3-Dihydroxy-3-methylpentanoate (in the Ile biosynthesis) (Figure C.2) (Singh and Shaner, 1995). Although KARI inhibitors have been found to be efficient inhibitors of their target, no KARI-inhibiting herbicide has been commercialized. *In vitro* experiments showed that different KARI inhibitors took so long to achieve substantial inhibition of their target (Dumas et al., 1994). Moreover, while AHAS is a low abundant enzyme present in plants, KARI is present in larger amounts, thus, higher concentrations of its inhibitors will be required to be as effective as AHAS inhibitors (Durner et al., 1993). Furthermore, it has been described that while AHAS inhibitors bind irreversibly to their target, KARI inhibitors binding seems to be reversible (Durner et al., 1993). Finally, AHAS inhibitors have been described to alter plant metabolism more dramatically than KARI inhibitors do (Zabalza et al., 2013).

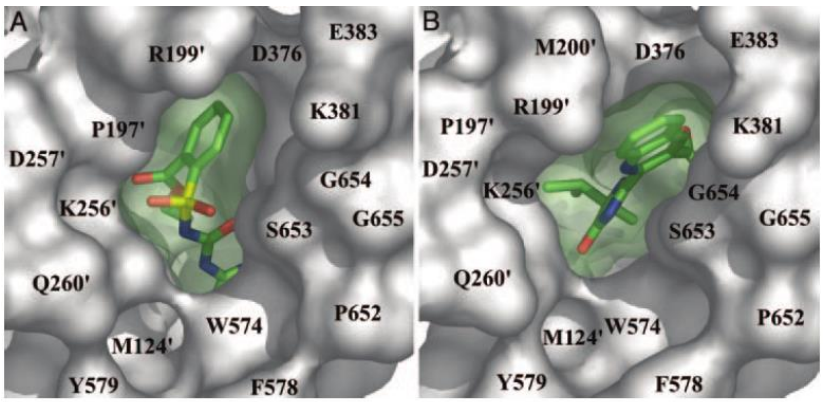


Figure C.3. Chlorimuron-ethyl (A) and imazaquin (B) (two acetohydroxyacid synthase (AHAS) inhibitors) position in the channel to the active site of *Arabidopsis thaliana* AHAS. Both herbicides are represented as stick models (carbon is green; nitrogen, blue; sulphur, yellow; oxygen, red; and chlorine is orange). The residues that line the channel are depicted as a grey surface. Note how both herbicides block the access to the active site of AHAS (McCourt et al., 2006).

AHAS is a nuclear-encoded enzyme that is transported to the plastids where the BCAA biosynthesis takes place (Singh and Shaner, 1995). It has both a catalytic subunit and a smaller regulatory subunit. In plants, this regulatory

subunit stimulates the activity of the catalytic site and confers sensitivity to feedback inhibition by BCAA (Duggleby et al., 2008). The catalytic subunit of AHAS is deep within a channel, and it has been described that AHAS inhibitors do not mimic the substrates of AHAS but bind to the substrate access channel, obstructing the substrate reach the catalytic site (Figure C.3) (McCourt et al., 2006).

The commercialized BCAA biosynthesis-inhibiting herbicides (AHAS inhibitors) belong to five chemical classes: sulfonylureas, imidazolinones, pyrimidinyl (thio) benzoates, triazolopyrimidines and sulfonylamino-carbonyl-triazolinones (Heap, 2016). There are 56 active ingredients belonging to the AHAS inhibitors (group B according HRAC classification).

C. 1. 1. Imidazolinones

After their introduction in the 1980s, the group of imidazolinones has become one of the most used herbicides worldwide. They control a wide spectrum of grass and broadleaf weeds, are highly selective, are effective at low application rates and present low toxicity for animals since they inhibit processes only present in microorganisms and plants. In order to reach they target, imidazolinones are absorbed through both leaves and roots, and then are transported to the meristematic tissues through the phloem or the xylem. In total, there are six available active ingredients belonging to the imidazolinones group (imazamethabenz-methyl, imazapic, imazapyr, imazethapyr, imazamox (IMX) and imazaquin), from which only IMX is permitted to be used in crops in Spain (Regulation (EC) Nº 1107/2009).

IMX is relatively persistent in soil, thus, it can affect sensitive rotational crops (O'Sullivan et al., 1998) and can decrease the soil microbial biomass content (Vischetti et al., 2002). Moreover, IMX has been shown to be easily leached, which can result in contaminated groundwater (Sakaliene et al., 2007).

C. 1. 2. Resistant weeds

Although imidazolinones are highly effective herbicides for weed management, only a few years after their introduction, resistant weeds began to emerge constituting a big problem for weed control in several crops. Currently, 156 species have been reported to present biotypes resistant to AHAS inhibitors, and indeed, they are the group to whom more resistant biotypes have been found (Figure B.1) (Heap, 2016).

The most generalized resistance mechanisms are the single point mutations in the target AHAS gene that reduce AHAS sensitivity; however NTSR occurs as well (Yu and Powles, 2014). Among the TSR, Tryptophan-574 to Leu and Proline-197 to Leu are the most common found mutations across the different resistant biotypes (amino acid numbering according to the *Arabidopsis thaliana* AHAS sequence) (Figure C.4) (Heap, 2016). On the other hand, the dominant NTSR mechanism has been found to be enhanced herbicide-metabolizing activity, often involving cytochrome P450 monooxygenases (Yu and Powles, 2014).

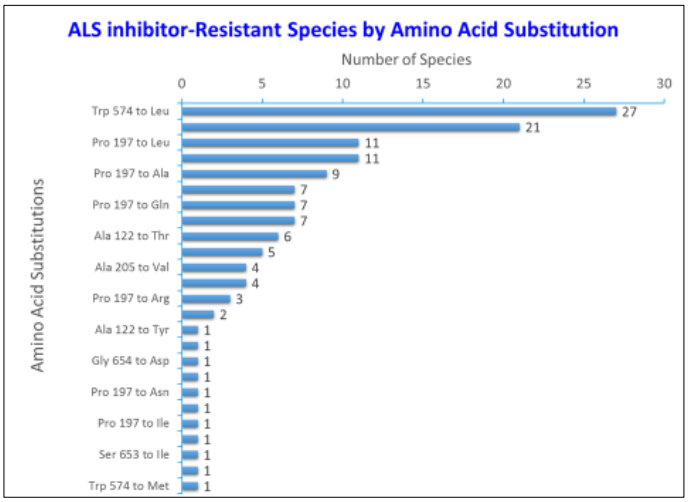


Figure C.4. Number of acetohydroxyacid synthase (AHAS) inhibitor-resistant species by most common amino acid substitutions in AHAS (Heap, 2016).

The introduction in the market of imidazolinone tolerant crops (named Clearfield Crops®) has increased the selection pressure for resistant weeds in the fields. These crops have been mutagenized and selected for resistance to AHAS inhibitors. Currently, imidazolinone tolerant maize, sunflower, wheat, rice, lentils and oilseed rape can be found in the market. The Clearfield Crops® permit the control of weeds difficult to control in the conventional crops, like the red rice in cultivated rice (Tan et al., 2005).

C. 2. Aromatic amino acid biosynthesis-inhibiting herbicides (Group G)

As it occurs for the BCAAs, animals cannot synthesize AAAs (tyrosine, phenylalanine and tryptophan) and they have to take them up with their diet. Besides, bacteria, archaea, fungi and plants are able for *de novo* AAA synthesis.

The AAA biosynthesis takes place in the plastids and it is divided into two parts. First, chorismate is synthesized in the shikimate pathway, a common route for the three AAAs, and second, the synthesis of the three AAAs branches in individual pathways for each AAA (Figure C.5). Under normal growth conditions, 20% of the carbon fixed by plants flows through the shikimate pathway (Rohr, 1993).

The first reaction in the shikimate pathway is the conversion of phosphoenolpyruvate (PEP) (derived from the glycolysis) and erythrose 4-phosphate (derived from the non-oxidative branch of the pentose phosphate pathway) to 3-deoxy-d-arabino-heptulosonate-7-phosphate in a reaction catalysed by the 3-deoxy-d-arabino-heptulosonate-7-phosphate synthase (DAHPS) (Figure C.5). DAHPS is key enzyme that links the primary carbon metabolism with the plant secondary metabolism, however, its role in the regulation of the flux between primary and secondary metabolism is still not known (Tzin and Galili, 2010).

Another key enzyme in the shikimate pathway is the EPSPS, whose inhibition is the target for the herbicide GLP (Steinrücken and Amrhein, 1980), the only known AAA biosynthesis-inhibiting herbicide (Figure C.1 and Figure

C.2). The EPSPS is a nuclear-encoded enzyme, but it functions in the plastid, where the AAA biosynthesis is located. The EPSPS catalyses the synthesis of the precursor of chorismate (EPSP) from PEP and shikimate 3-phosphate. GLP does not bind to the active site of the EPSPS, instead it seems to bind to a possible allosteric site, originating a structural change at the active site that prevents PEP binding (Cobb and Reade, 2010).

A wide variety of secondary metabolites are synthesized from AAAs, including hormones (e.g. auxin), hydroxycinnamic acids (e.g. sinapic acid and ferulic acid) and lignin (Figure C.5), indicating that AAAs not only function as building blocks for proteins. Besides, other secondary metabolites are also derived from different intermediates of the shikimate pathway (Figure C.5). As an example, quinate can be synthesized either from 3-dehydroquinate or shikimate, via quinate dehydrogenase (EC 1.1.1.24) or quinate hydrolyase, respectively (Bentley and Haslam, 1990; Leuschner et al., 1995). The physiological role of quinate has not been clarified, but it is considered as a reserve compound of the shikimate pathway that can re-enter the main trunk of this pathway by being converted either to 3-dehydroquinate or to shikimate by the above enzymes. Another example of the compounds that can be synthesized from the intermediates of the shikimate pathway are gallic acid and protocatechuic acid derived from 3-dehydroshikimate.

C. 2. 1. Glyphosate

GLP, the unique EPSPS-inhibiting commercialized herbicide, is absorbed through the leaves and it is translocated through the phloem to sink tissues following sucrose movement. It has a broad spectrum weed control, is non-selective and presents low soil residual activity, since it binds tightly to soil constituents preventing its movement to soil and groundwater (Reade and Cobb, 2002). Moreover, it has a short half-life because it can be easily degraded by soil microorganisms to aminophosphonic acid and glyoxylate (Van Eerd et al., 2003).

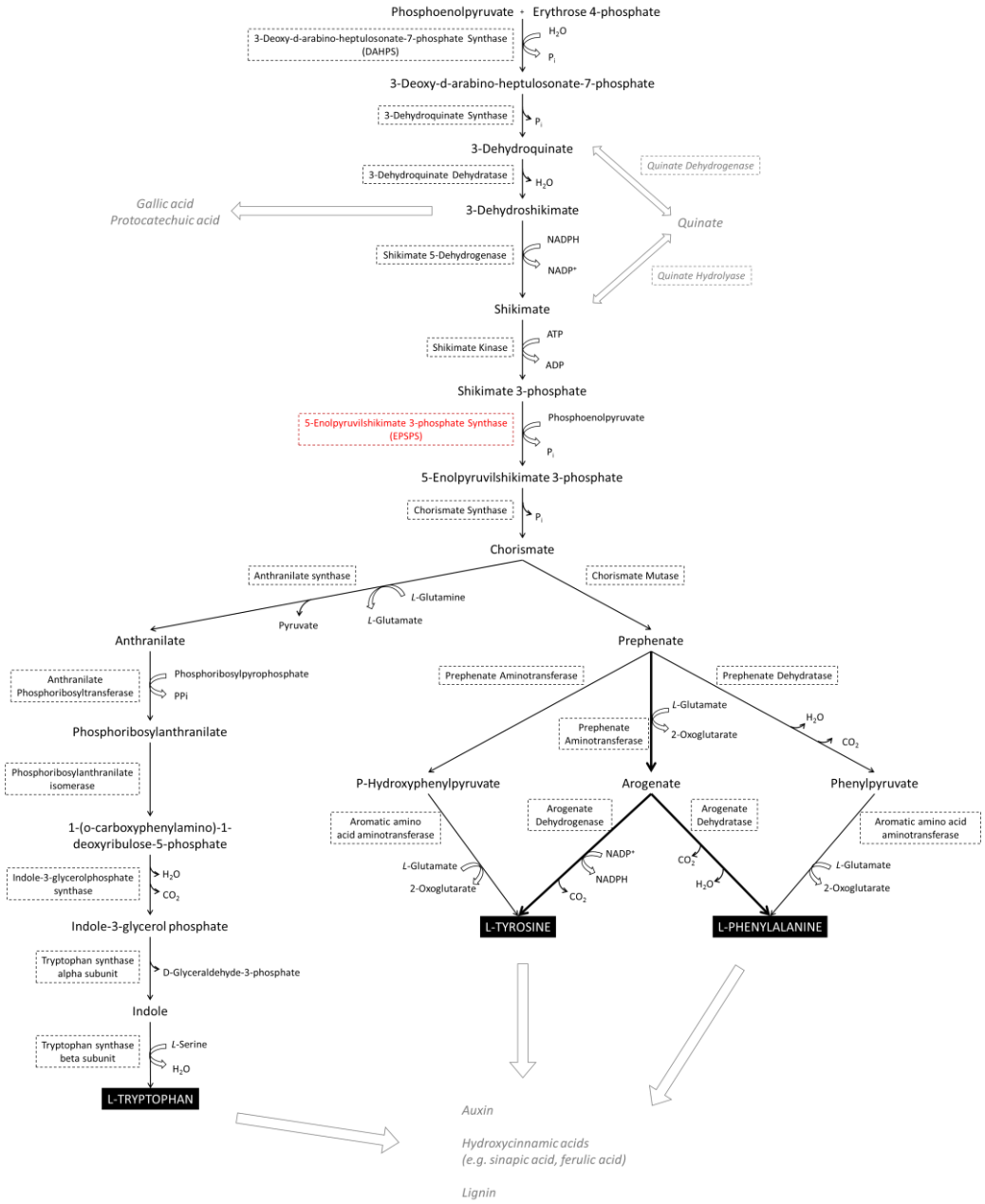


Figure C.5. Aromatic amino acid biosynthesis in plants. First, chorismate is synthesized in the shikimate pathway, and secondly the pathway is divided in individual branches for each aromatic amino acid. The secondary metabolites derived from this pathway are represented in grey. 5-Enolpyruvilshikimate-3-phosphate Synthase (EPSPS) (represented in red) is a key enzyme in the shikimate pathway that is specifically inhibited by the herbicide glyphosate.

C. 2. 2. Resistant weeds

After its commercial introduction in 1974, GLP has become the most worldwide used herbicide, but the use of this herbicide is now being threatened by the evolution of GLP-resistant weeds. Although the development of the first GLP-resistant weeds took some years (around 20 years), the number of resistant biotypes is increasing due to the high selection pressure present in the fields. To date, 32 species showing GLP-resistant biotypes have been reported (Figure C.6) (Heap, 2016).

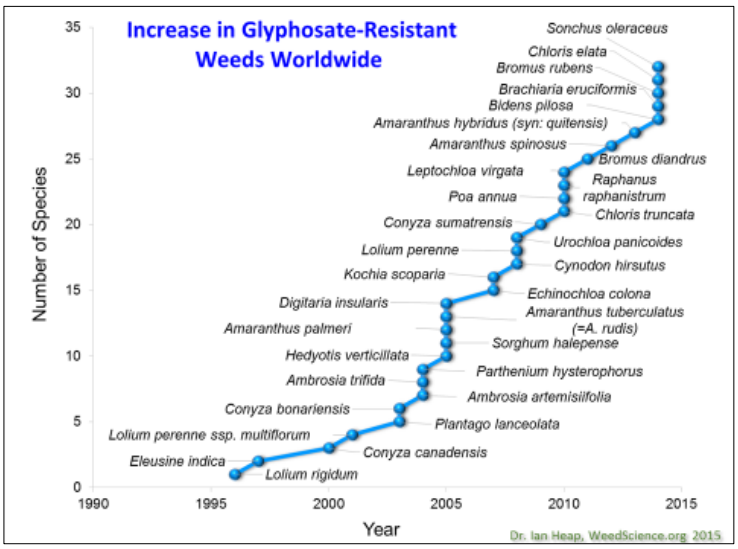


Figure C.6. Discovery of glyphosate-resistant species along the time (Heap, 2016).

Different TSR and NTSR mechanisms have been found to confer resistance to GLP. Two TSR mechanisms consisting of point mutations and overexpression of EPSPS gene have been reported in Italian ryegrass, Palmer amaranth or Rigid ryegrass, among others (Powles and Yu, 2010; Shaner et al., 2012). On the other hand, restricted herbicide leaf absorption and reduced translocation due to enhanced vacuolar sequestration have been shown to confer NTSR (Powles and Yu, 2010; Shaner et al., 2012).

The introduction of transgenic GLP-resistant crops in 1996 (known as Roundup Ready® Crops) increased GLP success. Currently, around 90% of the grown transgenic crops worldwide are GLP-resistant, and they include soybean, corn, cotton, canola, alfalfa. Three different techniques are applied in the development of the GLP-resistant crops:

1.- An *EPSPS* gene showing low sensitivity to GLP from *Agrobacterium* sp. strain CP4 (called CP4-*EPSPS*) together with the CaMV 35S promoter is inserted into the plant, to confer herbicide tolerance. The amino acid sequence of the GLP-tolerant CP4-EPSPS protein is around 50% similar and 30% identical to native EPSPS of plants and bacteria (Padgette et al., 1996; Dill, 2005). The vast majority of the commercial glyphosate-resistant crops contain the GLP-insensitive CP4-*EPSPS*.

2.- A gene coding for GLP oxidoreductase isolated from *Ochrobactrum anthropi* is also employed for GLP-resistant crops development. The GLP oxidoreductase degrades GLP to glyoxylate and aminophosphonic acid preventing the inactivation of the EPSPS (Dill, 2005). This detoxification mechanism is employed in combination with the introduction of GLP-insensitive *EPSPS* because the detoxification mechanism alone is not sufficient to produce plants resistant to the commercial GLP rates (Dill, 2005).

3.- A maize *EPSPS* transgene obtained through site-directed mutagenesis is also employed to produce GLP-resistant crops (Dill, 2005). This variant of maize EPSPS presents the threonine-102 to Ile and proline-106 to serine substitutions, mutations that confer commercial tolerance to GLP (Dill, 2005).

C. 3. Common physiological effects of BCAA and AAA biosynthesis-inhibiting herbicides

The target-site for both BCAA and AAA biosynthesis-inhibiting herbicides has been well described. However, it is not fully understood yet how AHAS or EPSPS inhibition leads to plant death. Although AHAS and EPSPS inhibitors act upon different pathways, several physiological common effects to both type of

herbicides have been reported recently (Orcaray et al., 2010; Orcaray et al., 2012), suggesting that both types of herbicides might provoke the plant death in a similar way.

C. 3. 1. Growth arrest and slow plant death

Both types of herbicides have been reported to cause a growth arrest that is followed by a slow plant death (Gruys and Sikorski, 1999; Wittenbach and Abell, 1999). Once they enter the plant, both types of herbicides are translocated and tend to accumulate in the meristematic tissues, where the first physical symptoms appear (Gruys and Sikorski, 1999; Wittenbach and Abell, 1999).

As a consequence of different types of imidazolinones or GLP application, a shoot and root growth arrest has been described in several plant species, such as, rice, thale cress, pea, soybean, common bean and common vetch (Gaston et al., 2003; Zabalza et al., 2007; Qian et al., 2009; Orcaray et al., 2010; Qian et al., 2011a; García-Garijo et al., 2012; García-Garijo et al., 2013). Moreover, sulfonylureas have also been shown to provoke an inhibition of the lateral root formation (Holmes et al., 2006).

C. 3. 2. Total free amino acid accumulation and total soluble protein content decrease

An accumulation in the free amino acid content has been reported in imazapyr (an imidazolinone)-treated corn (Shaner and Reider, 1986), in the leaves and the roots of imazethapyr-treated soybean (Zabalza et al., 2006) and pea (Zabalza et al., 2013), and in the shoot apical meristem of IMX-treated common bean plants (García-Garijo et al., 2012). Similarly, total free amino acid accumulation has been observed in pea plants treated with lethal doses of GLP (Orcaray et al., 2010).

As a consequence of AHAS or EPSPS inhibition, a decrease in the content of the amino acids whose biosynthesis is specifically inhibited is expected. However, after a transient decrease in the BCAA or AAA content, their content

increased in plants treated with AHAS or EPSPS inhibitors, respectively (Orcaray et al., 2010).

On the other side, a decrease in the total soluble protein content has been found in pea plants after AHAS or EPSPS inhibition (Zulet et al., 2013a). In this context, it has been suggested that after AHAS or EPSPS inhibition, proteolysis is enhanced in plants to degrade pre-existing proteins and to renew the BCAA or AAA levels for protein synthesis, which explains the increase in the total free amino acid content. Indeed, it has been shown that although protein synthesis exists after AHAS inhibition (Shaner and Reider, 1986), the amino acids that constitute the newly synthesized proteins do not come from the newly incorporated nitrogen but they would mainly be scavenged from already existing protein degradation (Zabalza et al., 2006). The proteolytic activities of ABIH-treated pea plants were investigated and although the expected increase in the proteolytic activities was not detected, several common effects were found after AHAS or EPSPS inhibition. Whereas the 26S proteasome system and the papain-like cysteine proteases were activated, the activities of the vacuolar processing enzymes, cysteine proteases and metacaspase 9 were decreased as a consequence of AHAS or EPSPS inhibition (Zulet et al., 2013a).

C. 3. 3. Net photosynthesis and stomatal conductance decline

A decrease in the net photosynthesis has been described in several plant species (e.g. pea, sugar beet and barley) after AHAS- or EPSPS-inhibiting herbicide application (Geiger et al., 1986; Royuela et al., 2000; Zabalza et al., 2004; Olesen and Cedergreen, 2010; Orcaray et al., 2010). Moreover, transcriptome profiling performed in rice treated with the R-imazethapyr enantiomer shown a decrease in the expression of photosynthesis-related genes (Qian et al., 2011b). The decline in the net photosynthesis has been related to a decrease in stomatal conductance (Fuchs et al., 2002; Zabalza et al., 2004).

C. 3. 4. Increase in the carbohydrate content

Total soluble sugars and starch have been shown to accumulate in the leaves and the roots of several plant species (e.g. pea and rice) after AHAS or EPSPS inhibition (Gaston et al., 2002; Zabalza et al., 2004; Orcaray et al., 2010; Qian et al., 2011b). In pea plants, the observed carbohydrate accumulation was independent of the site of application of the herbicide (sprayed onto the leaves or supplied to the nutrient solution) (Zabalza et al., 2004).

The growth arrest observed in ABIH-treated plants is not caused by a carbohydrate availability limitation. Instead, it seems that the available carbohydrates in the roots are metabolised at a lower-rate than they are being transported from the sources, which causes a decrease in sink strength that leads to an accumulation of these metabolites in the leaves (Zabalza et al., 2004; Orcaray et al., 2010). Indeed, a decrease in the gene transcripts involved in glycolysis and starch degradation was detected in imazethapyr treated rice (Qian et al., 2011b).

C. 3. 5. No changes in the total root respiration and increase of the alternative respiration pathway

Although more substrates are available for respiration in plants after ABIH application, in general, no significant increase in the total respiration rate has been found in plants treated with AHAS or EPSPS inhibitors (Gaston et al., 2003; Zabalza et al., 2011; Orcaray et al., 2012); although a transient increase in this parameter was detected after 2-3 days of AHAS-inhibiting herbicide application (Gaston et al., 2003; Zabalza et al., 2011).

In contrast, an increase in the alternative respiration capacity has been described in soybean after AHAS inhibition (Gaston et al., 2003) and in pea after IMX or GLP treatment (Armendáriz et al., 2015). Moreover, the transcript levels of the genes coding for one alternative oxidase increased in *A. thaliana* plants treated with imazapyr (Manabe et al., 2007) and in soybean after GLP treatment (Zhu et al., 2008).

It is not known what induces the alternative respiration in roots following AHAS or EPSPS inhibition, but this pathway has been described to be induced in plants exposed to several stresses (e.g., cold, phosphate starvation and pathogen infection) (Purvis and Shewfelt, 1993; González-Meler et al., 1999; Parsons et al., 1999; Simons et al., 1999), indicating that the alternative oxidase could have a function related to stress situations.

C. 3. 6. Induction of the ethanol fermentation

An increase in the activity and the content of the enzymes involved in the ethanol fermentation (pyruvate decarboxylase (PDC) (EC 1.2.4.1); alcohol dehydrogenase (ADH) (EC 1.1.1.1)) has been described in pea roots after AHAS or EPSPS inhibition (Gaston et al., 2002; Zabalza et al., 2005; Zabalza et al., 2011; Orcaray et al., 2012). On the other hand, fermentative metabolism activation has been observed in plants exposed to several stress conditions, when the oxygen is not limiting (Kimmerer and Kozlowski, 1982; Tadege et al., 1999). This suggests that the activation of ethanol fermentation is a general plant response to stress conditions.

Fermentative pathways are well-known to be induced in plants exposed to low-oxygen conditions, and fermentation has been described to contribute to low-oxygen stress tolerance, since the NAD^+ required for the glycolysis is regenerated in this pathway which ensures ATP production. However, the role of ethanol fermentation in plants in response to herbicide application is not understood. It can be proposed that the induction could be a plant defence mechanism helping to tolerate the herbicide, and/or, it could be a toxic consequence of the herbicidal activity, thus contributing to the chemical's toxicity.

Besides, while the regulation of the induction of ethanol fermentation has been deeply studied in plants exposed to low-oxygen conditions, the regulation of this pathway in plants treated with herbicides has not been clarified. One explanation for its induction might be that this pathway could metabolize the

pyruvate accumulated as a consequence of AHAS inhibition, since pyruvate is a common substrate for both PDC and AHAS. However, the induction of ethanol fermentation after EPSPS inhibition cannot be easily explained by this hypothesis since it is not a directly pyruvate-consuming enzyme.

C. 3. 7. Accumulation of secondary metabolites

As a consequence of EPSPS inhibition, an accumulation of shikimate has been described in the leaves of GLP-treated plants (Lydon and Duke, 1988; Hernandez et al., 1999; Orcaray et al., 2010). This provokes a deregulation in the carbon flow that results in the accumulation of other compounds upstream of the EPSPS inhibition point (e.g. protocatechuic and gallic acids), derived from intermediates of the shikimate pathway (Lydon and Duke, 1988; Hernandez et al., 1999; Orcaray et al., 2010).

Quinate, another metabolite derived from intermediates of the shikimate pathway (see Figure C.5 for biosynthetic pathway), has also been described to accumulate as a consequence of EPSPS inhibition (Orcaray et al., 2010). Interestingly, quinate has also been described to accumulate after AHAS inhibition and, moreover, it has been shown that exogenous quinate application can mimic the ABIH herbicidal effect (Orcaray et al., 2010), indicating that the accumulation of quinate as a consequence of ABIH application could contribute to the toxicity of this type of herbicides.

In addition to the effects on the quinate content, AHAS inhibitors have also been reported to provoke an accumulation of the metabolites derived from the AAAs. Indeed, an increase in the levels of many hydroxycinamic acids (p-coumaric acid, caffeic acid, ferulic acid, and sinapic acid) and in the lignin content has been described in different plants treated with several types of AHAS inhibitors (Suttle et al., 1983; Alla and Younis, 1995; Orcaray et al., 2011).

- GENERAL AIM -

GENERAL AIM

Among the herbicides whose site of action is the inhibition of the biosynthesis of amino acids, we can find those inhibiting branched-chain or aromatic amino acids. Although the target-site has been well-established for these two classes of herbicides, the contribution of the physiological side effects to the plant death is not fully-understood, which means that the exact reason why plant dies after herbicide application is not known.

Only after knowing more about the specific mechanisms by which plants die as a consequence of herbicide application, we could manufacture more potent herbicides to lower the commercial dosage, reduce the selection pressure for resistant weeds and minimize the negative effects on non-target organisms.

The general hypothesis of this work is that herbicides inhibiting two different pathways of amino acid biosynthesis (branched chain and aromatic) kill treated plants in a similar way. **Thus, the overall goal of this work is to gain further insights into the common toxicity of BCAA and AAA biosynthesis-inhibiting herbicides**. The thesis focuses in the pyruvate-consuming pathways that are induced after the application of both types of herbicides. Specifically, the induction of ethanol fermentations is studied in depth and new possible pyruvate-consuming pathways are evaluated.

Within this general objective, the following specific objectives were defined and addressed in three different chapters:

1.- To analyze the role of ethanol fermentation in the toxicity of ABIHs, two experimental approaches were established. The first one was conducted to analyze whether the application of low-oxygen stress to pea plants before herbicide treatment modifies the plant physiological response to the herbicide. The second one was performed with the use of a mutant line for the ethanol fermentation pathway. This objective is covered in Chapter 1.

2.- To investigate the regulation of the ethanol fermentation after herbicide treatment, the possible transcriptional regulation of ethanol fermentation and

the possible role of pyruvate as a signal is evaluated. This objective is covered in Chapter 2.

3.- To determine whether new pathways related to pyruvate are common to both types of herbicides, the possible role of the Aldehyde dehydrogenase gene family is evaluated. This objective is covered in Chapter 3.

Each chapter of this memory is organized into introduction, objectives, materials and methods, results, discussion (separated or combined in the same section) and conclusions. Then, there is a general abstract with the main conclusions of the present thesis. The bibliography is common for the three chapters and can be found at the end of the memory.

- CHAPTER 1 -

Role of the ethanol fermentation in the plant response after the application of herbicides that inhibit amino acid biosynthesis

1. 1. INTRODUCTION

1. 1. 1. Respiration in plants

The organic blocks on which plants depend for their survival are provided by the photosynthesis. This organic blocks are oxidized in the respiration and the energy stored in these carbon compounds, which is essential for the plant development and maintenance, is released. At the same time, many metabolic intermediates that are precursors for different processes (e.g. amino acid biosynthesis pathway) are generated during respiration. An overview of the respiration pathway in plants is presented in Figure 1.1.

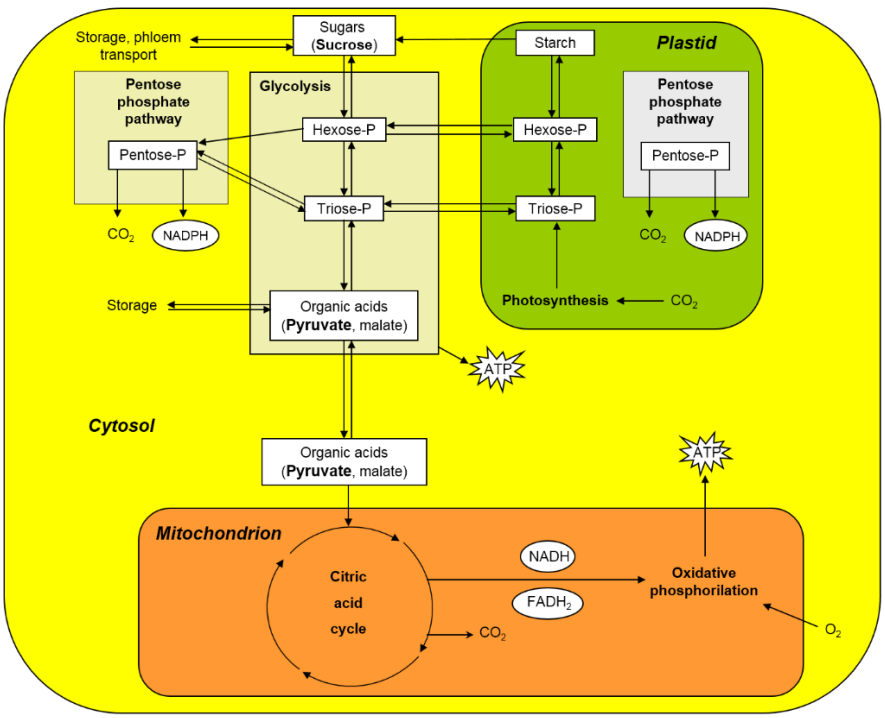


Figure 1.1. Overview of respiration in plants. Substrates for respiration are generated by other cellular processes and enter the respiration pathways. Glycolysis and the oxidative pentose pathways in the cytosol and plastids convert sugars into organic acids such as pyruvate, via hexose phosphates and triose phosphates, generating NADH or NADPH, and ATP. The organic acids are oxidized in the mitochondrial citric acid cycle, and the NADH and $FADH_2$ produced provide the energy for ATP synthesis by the electron transport chain and ATP synthase in the oxidative phosphorylation. In the gluconeogenesis, carbon from lipid breakdown is released in the glyoxysomes, metabolized in the citric acid cycle, and then used for the synthesis of sugars in the cytosol by reverse glycolysis (modified from Taiz and Zeiger, 2010).

Unlike in animals, in which glucose is the main respiration substrate, sucrose is the most common substrate for respiration in plants. However, there are other minor substrates that are generated in different pathways like the hexose phosphates and triose phosphates derived from starch degradation and photosynthesis, lipids and organic acids. These diverse substrates enter the respiration pathway at different points.

The first step in plant respiration is the degradation of sucrose, or the corresponding substrate, to produce the hexoses that are the initial point of the next step of the respiration, the glycolysis. In the glycolysis, the hexoses are oxidized and organic acids, mainly pyruvate and some malate, are produced. The glycolysis is localized in the cytosol and in this process reducing power (NADH) and a small amount of ATP are produced.

On the other hand, the pentose phosphate pathway provides more substrates for the glycolysis. In this process, which is located in both the cytosol and the plastids, glucose-6-phosphate is oxidized to ribulose-5-phosphate by the release of CO_2. Then, the ribulose-5-phosphate is converted into three to seven carbons containing sugars. During this pathway NADPH, which is necessary for the synthesis of nucleic acids and other compounds, is also produced.

In the next step of the respiration, the pyruvate (and malate) produced in the glycolysis enters the mitochondrion where it is completely oxidized to CO_2 in the citric acid cycle (also known as Krebs cycle or tricarboxylic acid (TCA) cycle). In this process a considerable amount of reducing power is also produced, in the forms of $NADH_2$ and $FADH_2$.

In the last step of the respiration, the oxidative phosphorylation, the NADH released during the different steps of the respiration (glycolysis, pentose phosphate pathway and citric acid cycle) is consumed and ATP is produced. Electrons from NADH are transferred along an electron transport chain localized in the inner mitochondrial membrane to the final acceptor, oxygen. This electron transfer releases a large amount of free energy, much of which is

conserved through the synthesis of ATP. This final stage completes the oxidation of sucrose (or the corresponding sugar).

1. 1. 2. Fermentation in plants

When the available oxygen is limiting, for example in plant roots exposed to flooding, neither the citric acid cycle nor the oxidative phosphorylation can function. Under this conditions, glycolysis can provide the ATP necessary for cell maintenance a since little amount of ATP is produced in this process. To regenerate the NAD^+ needed for glycolysis and to ensure ATP production, fermentation pathways are induced. Different fermentation pathways have been found in plants from which lactic fermentation and ethanol fermentation are the most important ones. Both pathways occur in the cytosol.

a) Lactic fermentation

In the lactic fermentation, the enzyme lactate dehydrogenase (LDH) (EC 1.1.1.27) reduces pyruvate to lactate in a single reaction that consumes NADH and thus, regenerates NAD^+ (Figure 1.2).

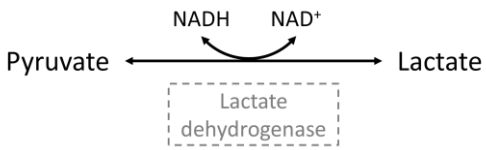


Figure 1.2. Overview of the lactic fermentation.

In some species (such as barley, maize and tomato) five active LDH tetrameric isoenzymes have been reported, which are composed by two subunits encoded by two genes (*LDH1* and *LDH2*) (Hoffman and Hanson, 1986; Christopher and Good, 1996). However, in other species (such as lettuce, *Capsella* and soybean) LDH consists of a single homotetrameric isoenzyme (Mulcahy and O'Carra, 1997). In *Arabidopsis*, LDH is encoded by a single gene (*LDH1*) which is located on chromosome 4 (At4g17260) (Dolferus et al., 2008).

b) Ethanol fermentation

There are two enzymes involved in the ethanol fermentation (Figure 1.3). First, the pyruvate is decarboxylated by the PDC to produce acetaldehyde, and subsequently, acetaldehyde is reduced via the oxidation of NADH and ethanol is produced in a reaction catalysed by the enzyme ADH.

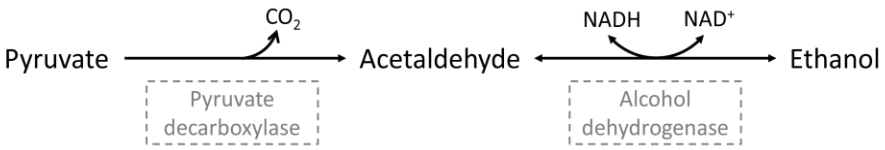


Figure 1.3. Overview of the ethanol fermentation.

PDC is encoded by a multigene family. In *Arabidopsis* PDC is encoded by four genes (Kürsteiner et al., 2003) which are highly homologous to each other and to other plant PDC genes. Among these genes, the expression of both *PDC1* and *PDC2* have been shown to induce during hypoxia and anoxia, and have been demonstrated to play an important role in the tolerance to submergence (Mithran et al., 2014). There are also four genes coding for PDC in rice, from which the expression of *PDC1*, *PDC2* and *PDC4* is induced by flooding and also participate in the tolerance to anoxia (Rivoal et al., 1990; Hossain et al., 1996).

In most of the flowering plants, there are two genes coding for the *ADH* gene, named, *ADH1* and *ADH2*, from which three dimeric isoenzymes are produced (Schwartz and Endo, 1966; Freeling, 1974; Tanksley and Jones, 1981; Xie and Wu, 1989; Gregerson et al., 1991). In pea, it has been observed that the three isoenzymes are induced in anaerobiosis (Llewellyn et al., 1987). By contrast, in *Arabidopsis* ADH is encoded by a single gene (Chang and Meyerowitz, 1986), and thus, a single isoform is present in this plant.

1. 1. 3. Fermentation in aerobic conditions

Apart of being an essential pathway in flooding tolerance, fermentation has been found to play a role in anther development and in the response to stress conditions when oxygen levels are not limiting (Tadege et al., 1999).

On the one hand, high levels of ADH protein have been detected in maize (Freeling and Bennett, 1985) and tobacco (Bucher et al., 1995) pollen. Aerobic fermentation in this tissue seems to be regulated by sugar availability rather than by oxygen concentration (Tadege and Kuhlemeier, 1997).

Besides, an induction of ethanol fermentation has been described in response to several abiotic and biotic stresses. An increase in the contents of acetaldehyde and ethanol (ethanol fermentation products) was detected in *Betula papyrifera* exposed to SO_2, ozone, water deficit and freezing stress, moreover, other woody and herbaceous plant species also increased the production of these metabolites in response to freezing (Kimmerer and Kozlowski, 1982; Kato-Noguchi and Yasuda, 2007).

The activity of ADH increased in maize seedlings exposed to osmotic stress (Kato-Noguchi, 2000), and the activity and transcript levels of this enzyme also increased in maize and rice seedlings exposed to low temperature conditions (Christie et al., 1991). Additionally, the expression of *ADH1* increased in *Arabidopsis* plants exposed to salinity, dehydration, low temperatures, osmotic stress and abscisic acid application (Dolferus et al., 1994; Kürsteiner et al., 2003) and in rice seedlings exposed to cold, water deficit, salinity and high temperatures (Minhas and Grover, 1999). It has been suggested that fermentation improves cold stress tolerance since maize *adh1-adh2* mutants were less tolerant to low temperature conditions comparing to the wild-type plants (Peters and Frenkel, 2004).

Regarding the PDC, an induction of *PDC1* has been detected in *Arabidopsis* plants exposed to salinity, low temperatures, osmotic stress and abscisic acid application (Kürsteiner et al., 2003).

An increase in the ethanol content and in the PDC activity was also detected in rice seedlings exposed to low temperature conditions (Kato-Noguchi and Yasuda, 2007).

1. 1. 4. Fermentation and herbicides

Fermentation has been reported also to be induced in plants treated with ABIHs. The activity and protein content of both enzymes involved in ethanol fermentation, PDC and ADH, increased in pea roots as a consequence of AHAS, EPSPS or GS inhibition (Gaston et al., 2002; Zabalza et al., 2005; Zabalza et al., 2011; Orcaray et al., 2012). Moreover, the activity of LDH also increased in plants treated with AHAS inhibitors (Gaston et al., 2002; Zabalza et al., 2005) although it did not increase as a consequence of EPSPS inhibition (unpublished data).

In low-oxygen conditions induction of fermentation allows to maintain the energy status of the cells, since the mitochondrial respiration, which is the main pathway for ATP synthesis, does not function in the absence of oxygen. This way, fermentation can provide little amount of ATP for cell maintenance. However, no decrease in the total respiration has been found in the roots of pea plants treated with AHAS or EPSPS inhibitors and also, the adenylate energy charge did not drop (Gaston et al., 2003; Zabalza et al., 2011; Orcaray et al., 2012). Thus, fermentation is induced in plants in response to ABIHs even though the energy status of the cells is not affected.

Fermentation has been described to be induced in plants exposed to different stresses even when oxygen is not limiting, including ABIH application. However, although fermentation in plants exposed to low-oxygen conditions has been deeply studied, its role in plants exposed to other stresses when oxygen is not limiting has not been explained yet. Two, non-contradictory explanations can be considered. Firstly, the induction of these two pathways could be a plant defence mechanism that promotes better tolerance of the herbicide. Secondly, it could be a consequence of the herbicidal activity, thus contributing to the chemical's toxicity. Since during fermentation ethanol and lactate, metabolites

shown to be toxic for the plants (Perata and Alpi, 1993), are produced, fermentation could be related to the toxicity of the herbicide.

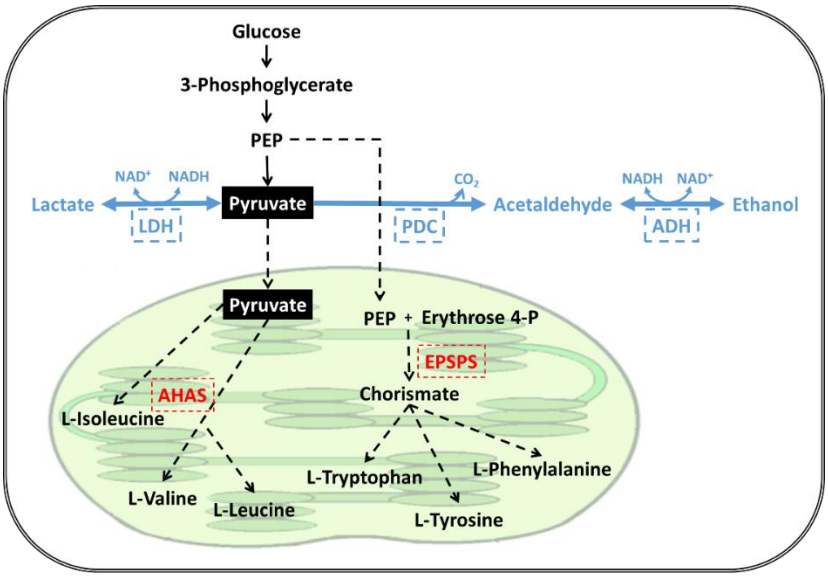


Figure 1.4. Overview of the cellular localization of the target-sites of herbicides inhibiting amino acid biosynthesis (shown in red) and simplified view of the branched-chain and aromatic amino acid biosynthesis. Pyruvate plays a central role in linking fermentative metabolism (shown in blue) and amino acid biosynthesis. ADH, alcohol dehydrogenase; AHAS, acetohydroxyacid synthase; EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase; LDH, lactate dehydrogenase; PDC, pyruvate decarboxylase; PEP, phosphoenolpyruvate.

1. 2. OBJECTIVES

Currently, the use of ABIH is of great importance in weed management. It has been described that although they inhibit different amino acid biosynthesis pathways, some common physiological effects are triggered as a consequence of ABIH application in plants. One of the common physiological consequences described in plants is that an induction of ethanol fermentation is triggered after AHAS or EPSPS inhibition. Moreover, ethanol fermentation has been shown to be induced in plants exposed to different abiotic stresses, such as, low temperatures, SO_2 or drought. However, the role of fermentation in the response of the plants to these stress conditions has not been clarified yet.

The main objective of the first chapter of the present thesis is **to evaluate the importance of the ethanol fermentation in the response of the plants to AHAS and EPSPS inhibitors,** trying to outline if fermentation induction is related to the toxicity of the herbicides or it is a plant defence mechanism that alleviates the herbicide effect. This general aim was approached by two specific objectives and two experimental approaches that are individually presented:

1. To evaluate whether the induction of the ethanol fermentation pathway before the ABIH application modifies the plant response to the herbicide, pea plants were exposed to two different times of hypoxia before herbicide application in order to induce the ethanol fermentation pathway. Then, an AHAS inhibitor was applied to the nutrient solution and the more characteristic physiological effects triggered by ABIH application were evaluated. This study is included in the PART I of this Chapter.
2. To evaluate whether the lack of the ethanol fermentation pathway modifies the response of the plants to ABIH application, *Arabidopsis thaliana* mutant plants lacking the *ADH1* gene were treated with AHAS or EPSPS inhibitors and the more characteristic physiological effects provoked by ABIH application were evaluated. This study is included in the PART II of this Chapter.

1. 3. MATERIALS AND METHODS

1. 3. 1. Plant material and treatment application

a) *Pisum sativum*

Pisum sativum L. cv. snap sugar boys where surface sterilized according to Labhilili et al. (1995). First, they were placed in a 1% (w/v) sodium hypochlorite and 0.01% (w/v) SDS mixture containing solution for 40 min and rinsed several times with deionized water. Then, they were soaked in 0.1 N HCl for 10 min and rinsed again with deionized water several times. For germination, the seeds were sown in trays containing moist perlite:vermiculite (1:1, v/v) and placed at 26°C for 96 h in darkness. Once germinated, the plants were transferred to 2.7 L tanks (15 plants per tank) filled with the nutrient solution described in Rigaud and Puppo (1975), substituting KH_2PO_4 by K_2HPO_4 and enriched with 10 mM KNO_3. The final composition of the nutrient solution was as follows: 1,1 mM K_2HPO_4, 0,8 mM $MgSO_4 \cdot 7H_2O$, 2,7 mM KCl, 0,1 mM $Na_2FeEDTA$, 0,7 mM $CaSO_4 \cdot 2H_2O$, 16,5 µM $Na_2MoO_4 \cdot 2H_2O$, 3,7 µM $FeCl_3 \cdot 6H_2O$, 3,5 µM $ZnSO_4 \cdot 7H_2O$, 16,2 µM H_3BO_3, 0,5 µM $MnSO_4 \cdot H_2O$, 0,1 µM $CuSO_4 \cdot 5H_2O$, 0,2 µM $AlCl_3 \cdot 6H_2O$, 0,1 µM $NiCl_2 \cdot 6H_2O$, 0,1 µM KI. The pH was adjusted to 7.6 and the nutrient solution was replaced once a week. The plants were grown in a growth chamber under the following conditions: 12/12 h day/night cycle photoperiod; 600 µmol m^{-2} s^{-1} light, 60-70% relative humidity and 20°C/16°C day/night temperature. To prevent roots from hypoxia, the nutrient solution was continuously aerated.

When the plants were 12-days-old, they were separated in three groups (Table 1.1). In order to obtain low-oxygen conditions for the induction of fermentation, the aeration was removed in two of the groups for 48 hours (group named Hypoxia-48), and for 24 hours in another two groups (group Hypoxia-24). The other two groups were maintained continuously aerated (group No-Hypoxia). After the 48 or 24 h the tanks were again aerated, this day was considered as the day 0. The nutrient solution was replaced the day 0 in all the tanks and the herbicide was applied to half of the tanks from the group Hypoxia-48 (named IMX-48), half of the plants from the group Hypoxia-24 (named IMX-

24), and to half of the tanks from the group No-Hypoxia (named IMX-0). The herbicide was applied to the nutrient solution as commercial formulation at a final concentration of 5 mg active ingredient L^{-1} (16.33 μM) of IMX (Pulsar®40, BASF Española SA, Barcelona, Spain). The other half of the plants from the groups Hypoxia-48, Hypoxia-24 and No-Hypoxia were not treated with herbicide and were the control of each group for the comparison with the herbicide treated plants. They were named as HYP-48, HYP-24 and C-0, respectively. The experiment was performed in duplicate. The applied herbicide doses provoked plant death in 20 days.

GROUP	ABBREVIATURE	TREATMENT DESCRIPTION
No-Hypoxia	C-0	No treatment was applied.
	IMX-0	Application of 5 mg L^{-1} of imazamox at day 0.
Hypoxia-24	HYP-24	The aeration was removed for 24 h before the day 0. Aeration was again placed at day 0 until the end of the experiment. No herbicide was applied.
	IMX-24	The aeration was removed for 24 h before the day 0. The aeration was again placed at day 0 until the end of the experiment. At day 0 imazamox was applied at a final concentration of 5 mg L^{-1}.
Hypoxia-48	HYP-48	The aeration was removed for 48 h before the day 0. Aeration was again placed at day 0 until the end of the experiment. No herbicide was applied.
	IMX-48	The aeration was removed for 48 h before the day 0. Aeration was again placed at day 0 until the end of the experiment. The day 0 imazamox was applied at a final concentration of 5 mg L^{-1}.

Table 1.1. Summary of the six different treatments.

For the analytical measurements, intact root samples were taken at day 0, before herbicide application, and at days 1, 3 and 7 after IMX application, this time points were chosen in order to allow us to evaluate physiological and

biochemical plant responses induced by the herbicide but not directly resulting from cell death.

Plant material was immediately frozen in liquid nitrogen and stored at -80°C for further analysis. Later, frozen samples were ground under liquid nitrogen using a Retsch mixer mill (MM200, Retsch®, Haan, Germany), the needed amount of tissue for each analysis was separated and stored at -80°C. Some fresh material was dried for 48 h at 80°C in order to obtain the fresh weight/dry weight ratio.

To ascertain that the wanted low-oxygen conditions were obtained the oxygen concentration in the nutrient solution was monitored (Figure 1.5.) with a portable oxygen meter OXI 45 (HACH LANGE SPAIN, S.L.U., Barcelona, Spain). When the aeration was removed (indicated with a black arrow for the Hypoxia-48 group and grey arrow for the Hypoxia-24 group in Figure 1.5) the oxygen concentration in the nutrient solution drastically decreased. At day 0, the oxygen concentration present in the nutrient solution of the tanks from the groups Hypoxia-48 and Hypoxia-24 was about 30-40%. By contrast, in the tanks that were continuously aerated (group No-Hypoxia) the oxygen concentration maintained at around 100%. These results indicate that the desired conditions to carry out the experiment were obtained.

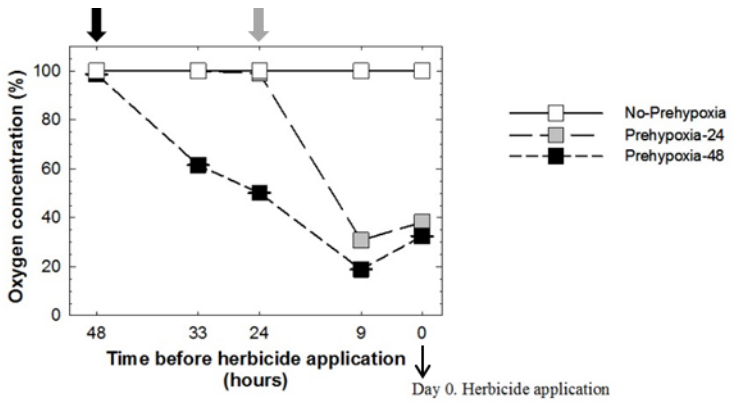


Figure 1.5. Oxygen concentration (%) in the nutrient solution before herbicide application. Black and grey arrows in the top of the graph indicate aeration removal 48 h and 24 h before herbicide application, respectively.

b) *Arabidopsis thaliana*

A. thaliana ecotype Columbia-0 (Col-0) was used as the wild-type control. The *A. thaliana* Col-0 T-DNA mutants defective for *ADH1* (NASC ref. N552699, Banti et al., 2008) were kindly provided by Prof. van Dongen (Institute of Biology, RWTH Aachen University, Germany).

b. 1) Hydroponic system

Plant seeds were surface sterilized with 70% (v/v) ethanol for 2 min and with 10% (v/v) sodium hypochlorite for 15 min and rinsed several times with sterile deionized water before sowing them on Seedholders (Araponics SA, Belgium) filled with 0.65% (w/v) plant agar. Twelve seedholders were placed in each 1.7 L tanks filled with deionized water and the tanks were placed for 3 d at 4°C in darkness before they were transferred to the growing chamber. Plants were grown under 120-150 µmol m^{-2} s^{-1} light, 65% relative humidity, at 23°C/18°C day/night temperature. The day/night cycle consisted of 12/12 h for the first 4 weeks and 8/16 h day/night photoperiod afterwards to prevent flowering. One week after placing the seeds on Seedholders (Araponics SA, Belgium), the water of the tanks was replaced with nutrient solution. The nutrient solution was slightly modified from Loqué et al., 2003: 1 mM NH_4NO_3, 1 mM KH_2PO_4, 1 mM $MgSO_4 \cdot 7H_2O$, 250 µM $CaCl_2 \cdot 2H_2O$, 0.1 mM Na-Fe-EDTA, 50 µM KCl, 0.1 mM H_3BO_3, 0.01 mM $MnSO_4 \cdot H_2O$, 2 µM $ZnSO_4 \cdot 7H_2O$, 1.5 µM $CuSO_4 \cdot 5H_2O$, and 0.1 µM $Na_2MoO_4 \cdot 2H_2O$. The nutrient solution was replaced every week. Aeration was set in the tanks when the plants were six-week-old and maintained onward.

When plants were approximately 8-week-old, herbicides were applied to the nutrient solution. The two herbicides were applied as commercial formulations at a final concentration of 1.5 mg active ingredient L^{-1} (4.9 µM) of IMX (Pulsar®40, BASF Española SA, Barcelona, Spain) or 20 mg active ingredient L^{-1} (87.65 µM) of GLP (Glyfos®, Bayer CropScience, S.L, Paterna, Valencia, Spain). The experiment was performed in triplicate.

Preliminary studies were conducted to find comparable doses of IMX and GLP causing similar effects and provoking death (photosynthesis almost zero) within 20 days, as in pea.

Net carbon dioxide assimilation rates were measured from the youngest, fully expanded leaf in intact plants using a portable ADC-LCpro+ system equipped with an *Arabidopsis* chamber (ADC BioScientific Ltd., Herts, UK). Measurements were made in the growth chamber under growing conditions (400 ppm CO_2, 25°C leaf temperature, 1.1 kPa VPD).

For the analytical determinations samples were taken after three days of herbicide application, before obvious visual plant death was observed. This time point was chosen in order to allow us to evaluate physiological and biochemical plant responses induced by the herbicide but not directly resulting from cell death.

Intact leaf and root samples were taken were immediately frozen in liquid nitrogen and stored at -80°C for further analysis. Later, frozen samples were ground under liquid nitrogen using a Retsch mixer mill (MM200, Retsch®, Haan, Germany), the needed amount of tissue for each analysis was separated and stored at -80°C.

b. 2) Vertically grown plants on agar containing plates

Seeds were sterilized as explained in section 1.3.1.b.1 (using sterilized material and reactives) and then were transferred to petri dishes plates containing 10 mL of 0.1% (w/v) plant agar. Plants were incubated for 3 days at 4°C in darkness before they were transferred to the growing chamber. Plants were grown under 120-150 μmol m^{-2} s^{-1} light, 65% relative humidity at 23°C/18°C day/night temperature and 12/12h day/night cycle. After 4 days in the growing chamber, when the root length was about 1 cm, seedlings were transferred to 12 x 12 cm plates containing 50 mL of half-strength Murashige and Skoog (MS) medium (pH 5.8), 1% sucrose (w/v) and 0.1% (w/v) plant agar (10 seeds per plate) and, where corresponding, the selected herbicide dose. The

two herbicides were sterilized using 0.12 µm filters and were added to the medium before its solidification.

Since the applied herbicide dose in the hydroponically grown *A. thaliana* plants was too high for this growing method (plants died within two days), preliminary studies were conducted to find a herbicide dose that was not too aggressive and killed the plant in few days, but that was sufficiently aggressive to have an effect on the plant (growth). Herbicides were applied as commercial formulations, and 0.005 mg active ingredient L^{-1} of IMX (0.016 µM) (Pulsar®40, BASF Española SA, Barcelona, Spain) and 0.25 mg active ingredient L^{-1} of GLP (1.1 µM) (Glyfos®, Bayer CropScience, S.L, Paterna, Valencia, Spain) were chosen as herbicide doses. Other plants were not treated with herbicide and were the control plants.

1. 3. 2. Growth parameters

a) *Pisum sativum*

To monitor the growth of the plants, the shoot and the root lengths were measured in hydroponically grown pea plants the days 0, 1, 3 and 7.

b) *Arabidopsis thaliana*

Since no growth effect can be detected in the hydroponically grown plants because they were old and had reached the growing plateau, in order to monitor the herbicide effect on the growth of *A. thaliana* plants, seedlings were grown on agar containing vertical plates as described in section 1.3.1.b.2 of the present chapter. Pictures were taken to monitor seedlings growth.

1. 3. 3. PDC and ADH activities and soluble protein content

PDC and ADH activities were assayed in ground leaf and root samples. About 0.1g FW of frozen samples were homogenized in the following extraction buffer (4 mL per g FW for pea and 2.5 mL per g FW for *A. thaliana*): 50 mM MOPS (pH 7.0), 5 mM $MgCl_2$, 1 mM Na_2-EDTA, 20 mM KCl, 10 mM DTT and 0.08% (w/v) β-mercaptoethanol. Homogenates were centrifuged for 30 min at 18,000 *g* and 4°C; and, after centrifugation, supernatants were collected in new

tubes for the determination of the soluble protein content and the *in vitro* activities of PDC and ADH.

For the enzyme activity assays the supernatant was desalted in a 96-well of 800 µL Whatman UNIFILTER™ filtration microplate (Whatman Inc., Clifton, NJ, USA) containing 600 µL of Sephadex® G-50. The columns were equilibrated with a desalting buffer (50 mM MOPS (pH 7.0), 5 mM $MgCl_2$ and 20 mM KCl).

Twenty microlitres of the desalted supernatant were taken to measure the enzymatic activities in a total reaction volume of 170 µL. The reactions were monitored at 30°C for 10 min at a 340 nm in a Sinergy™ HT Multi-Detection Microplate Reader (BioTek Instruments Inc., Winooski, VT, USA).

PDC activity was measured in the pyruvate-to-acetaldehyde direction in the presence of 25 mM oxamate to inhibit LDH (Bouny and Saglio, 1996). The reaction cocktail contained: 100mM Tricine (pH 6.5), 2 mM $MgCl_2$, 1 mM DTT, 0.2 mM NADH, 3 U ml^{-1} *Saccaromyces cerevisae*'s ADH, 25 mM oxamate, 1 mM TPP and 10mM pyruvate. Consumption of NADH was monitored following the reduction in absorbance at 340 nm. For each sample, a measurement without substrate was done.

ADH activity was assayed in the ethanol-to-acetaldehyde direction according to John and Greenway (1976). The reaction cocktail contained: 50 mM Bicine (pH 8.8), 5 mM $MgCl_2$ and 49 µM absolute ethanol and 1 mM NAD^+. Production of NADH was monitored following the increase in absorbance at 340 nm. For each sample, a measurement without substrate was done.

The soluble protein content was monitored as Bradford (1976) in the crude and the desalted supernatant. Protein aliquots were diluted with deionized water to 1:60 for the leaves and to 1:30 for the roots and 60 µL of the dilutions were mixed with 200 µL BioRad Protein Assay Dye Reagent (Bio-Rad Laboratories Inc., Hercules, CA, USA). The suspensions were incubated at room temperature for 5 min and the OD was measured at 595 nm. The quantification of the protein aliquots was calculated by calibrating the measurement of each

sample with the OD (595 nm) against a given concentration (0.1 μg μL^{-1}) of standard proteins (BSA) curve ranging from 0 μg to 6 μg. For the absorbance measurements a Sinergy™ HT Multi-Detection Microplate Reader (BioTek Instruments Inc., Winooski, VT, USA) was used.

1. 3. 4. In gel ADH activity

Desalted extracts were obtained from pea roots as described in 1.3.3 but 2.5 mL extraction buffer per 0.1 g FW were used in the initial step. Due to material availability no samples of the treatments HYP-48 and IMX-48 were used.

Native electrophoresis was run in a 12.5% polyacrylamide gel (Phast Gel® Homogeneous 12.5% in 0.112 M Acetate, 0.112 M Tris (pH 6.4)) at 4°C in a Phast System™ (Pharmacia, LKB, Biotechnology AB, Uppsala, Sweden). Phast Gel® Buffer Strips Native (0.88 M L-Alanine, 0.25 M Tris (pH 8.8)) were used for the electrophoresis. In each line 1.95 μg of protein were loaded.

ADH specific staining was performed as described in (Schwartz and Endo, 1966) with minor modifications. The gel was incubated in darkness for 15 min in a solution composed of 25 mM Tris-Cl (pH 8), 0.8% (v / v) ethanol, 0.144 mM nitro blue tetrazolium, 0.65 mM phenazine methosulfate, and 0.24 mM NAD^+.

1. 3. 5. SDS-PAGE and immunodetection of PDC and ADH

Protein extracts for immunoblot determinations were obtained from previously ground pea root samples as described in section 1.3.3, except that two mL of extraction buffer were used per 0.1 g FW root samples and extracts were not desalted. Since not enough root samples from group Hypoxia-48 were available for all the determinations, no immunoblot determinations were done for the plants of this group (HYP-48 and IMX-48).

Equal amounts of proteins (30 μg) were loaded and they were separated using 1 mm thick 7.5% (w/v) polyacrylamide resolving gel and a 4.6% (w/v) polyacrylamide stacking gel in a vertical electrophoresis cell (Mini Protean III; Bio-Rad Laboratories Inc., Hercules, CA, USA) at 150 V for 60 min. Gels were

blotted onto PVDF membranes for 75 min at 100 V in a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad Laboratories Inc., Hercules, CA, USA). After transfer, membranes were blocked with 5% (w/v) non-fat milk powder in 10% Tween Tris Buffer Saline (TTBS) for one hour and washed three times using 10% TTBS. Blots were incubated overnight at 4°C with the corresponding antibody. PDC and ADH antibodies were used at dilutions of 1:1,000 and 1:500, respectively. The PDC antibody was kindly provided by Dr. König (Martin Luther University) and it was raised in rabbits against PDC enzyme purified from germinating pea seeds (Mücke et al., 1995). The ADH antibody was produced by a custom peptide facility (Biogenes, Berlin, Germany) and a short conjugated peptide as antigen (C-KGTFYGNYKPRTDL-COOH). The antibody was raised in rabbit using standard protocols from the manufacturer.

After primary antibody incubation, membrane was washed three times with 10% TTBS and further incubated with goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase (1:20,000; Sigma-Aldrich Co., St. Louis, MO, USA) for 1 h. After another round of washing, cross-reacting protein bands were visualized using the Amplified Alkaline Phosphatase Goat Anti-Rabbit Immun-Blot® Assay Kit (Bio-Rad Inc., Hercules, CA, USA) according to manufacturer instructions. The intensity of the bands was quantified using a GS-800 densitometer (Bio-Rad Inc., Hercules, CA, USA).

1. 3. 6. Carbohydrate Extraction and Determination

Ethanol soluble sugars (glucose, sucrose and fructose) were extracted from about 0.05 g of previously ground leaves and roots in 1.5 mL of 80% ethanol. Samples were sonicated for 25 min at 30°C in an ultrasonic bath and the tubes were centrifuged at 7,500 *g* for 5 min at 4 ªC. Supernatant was transferred to a new tube and the pellet was washed two more times as above. All the collected supernatant was dried in a Turbovap® LV Evaporator (Zymark, Hopkinton, MA, USA) at 40°C and 1.2 bar. When all the ethanol was evaporated, the dried sample was suspended in 1 mL of deionized water, mixed and centrifuged at

6,000 *g* for 10 min at 4°C. The supernatant was collected in a new tube and stored at -20°C until its utilization.

The pellet obtained after the extraction of ethanol soluble sugars was used for starch extraction as described by MacRae (1971). After drying the pellet at 70°C for 24 h, it was suspended in 1 mL of deionized water. Then, the tubes were boiled at 100°C for 1 h in a water bath, and after cooling the tubes on ice, 250 µL of 0.082% (w/v) amyloglucosidase dissolved in 8.55 mM acetate (pH 4.5) was added. The amyloglucosidase enzyme catalyses the hydrolysis of starch to its monomers of glucose. The reaction was incubated at 50°C overnight in darkness continuously shaking. Then, the mixture was centrifuged at 7,500 *g* for 15 min at 4°C and the supernatant was collected and stored at -20°C until it was used. The starch content was measured as glucose content.

The contents of fructose, glucose, and sucrose from the ethanol soluble fraction and glucose content coming from the hydrolysis of the starch were analysed by high-performance capillary electrophoresis in a P/ACE™ MDQ (Beckman Coulter Inc., Brea, CA, USA) according to Warren and Adams (2000). The background buffer consisted of 10 mM benzoate (pH 12.0) and 0.5 mM myristyltrimethylammonium bromide (MTAB). The applied potential was -15 kV, and the capillary tubing was 50 µm internal diameter and 31.4/38.4 cm long. The indirect UV detection wavelength was set at 225 nm.

Soluble sugars were expressed as milligrams per gram of DW, and starch was expressed as milligrams of glucose per gram of DW.

1. 3. 7. Free Amino Acid Extraction and Determination - Amino acid and glutathione content determination

a) Total free amino acid extraction and determination – pea plants

Total free amino acid pool was measured in the roots of pea plants according to the method proposed by Yemm and Cocking (1955).

The same extracts obtained for ethanol soluble sugars determination (described in section 1.3.6) were used. Twenty µL of the extracts were mixed

with 500 µL of citrate buffer (0.88 M citric acid + 1.6 M NaOH) and 430 µL ninhydrin reactive (1.25 g ninhydrin in 125 mL 2-methoxyethanol + 50 mg ascorbic acid in 5 mL H_2O). Samples were boiled at 100°C for 20 min and after cooling 1 mL of ethanol 60% was added to the tubes and mixed vigorously. The absorbance of the mixture was measured at 570 nm in a Sinergy™ HT Multi-Detection Microplate Reader (BioTek Instruments Inc., Winooski, VT, USA). A glycine standard was used, and the results were expressed as mmol glycine per g^{-1} DW.

b) Free amino acid extraction and determination – *Arabidopsis thaliana* plants

Total free amino acids were measured from ground leaf or root samples by adding 1.5 mL of 1 M HCl to about 0.1 g FW of plant tissues. After incubating the tubes for 10 min on ice, they were centrifuged at 18,000 *g* for 15 min at 4°C. The supernatants were transferred to new tubes and neutralized to 7.0 - 8.0 pH with NaOH. The extracts were kept at -20°C until further analysis.

For the free amino acids determination (except cysteine), known concentrations of internal standards norvaline and homoglutamic acid were added to the mixture. Samples were then derivatized with 1 mM fluorescein isothiocyanate dissolved in acetone and the homogenates were 5-fold diluted in 20 mM borate buffer (pH 10.0). The mixtures were incubated for 15 h at room temperature in the dark.

The content of free amino acids was determined using a Beckman Coulter capillary electrophoresis PA-800 (Beckman Coulter Inc., Brea, CA, USA) coupled to laser-induced fluorescence detection (argon laser at 488 nm). The separation was mainly as described by Takizawa and Nakamura (1998) and by Arlt et al. (2001). A fused-silica capillary with a length of 43/53.2 cm and 50 µm internal diameter was employed. For amino acid separation, 45 mM α-cyclodextrin in 80 mM borax buffer (pH 9.2) was used. Analyses were performed at 20°C and at a voltage of +30 kV except for Valine and Tryptophan determinations were the applied voltage was +20 kV.

1. 3. 8. Cysteine and Glutathione content

Cysteine and glutathione content were determined from the same acid extracts described in section 1.3.7.b. Samples were derivatized with 5-iodoacetamide fluorescein and known concentrations of N-acetylcysteine were added to the samples as internal standard. For the determination of the total content of cysteine and glutathione samples were reduced with tributylphosphine.

Capillary electrophoresis was performed using a Beckman Coulter capillary electrophoresis PA-800 (Beckman Coulter Inc., Brea, CA, USA). A fused-silica capillary with a length of 50/60.2 cm and 50 μm internal diameter was employed. For metabolite separation, 20 mM de Na_3PO_4, 16.5 mM H_3BO_3, 100 mM N-methyl N-glucamine (pH 11.2) buffer was used. Analyses were performed at 25°C and at a voltage of +30 kV. Fluorescein was detected using a laser at 494 nm excitation and 518 nm emission.

1. 3. 9. Organic Acid Extraction and Determination

a) Long Chain Organic Acid Extraction and Determination

Long Chain Organic Acids were extracted from *A. thaliana* leaves and roots as described in section 1.3.6.

Metabolite levels were analysed by ion chromatography in a DX-500 System (Dionex Corporation, Sunnyvale, CA, USA) by gradient separation with Dionex Ion pack AG11+AS11 columns (from 0.2 mM NaOH to 45 mM NaOH and from 10% of methanol to 20% of methanol, in 27 min, at a flux of 1 mL min^{-1}).

b) Short Chain Organic Acid Extraction and Determination

Short chain organic acids were extracted from previously ground *A. thaliana* leaf and root samples.

About 0.1 g of plant samples were homogenized in 0.5 mL 1M HCl. Tubes were centrifuged at 18,000 *g* for 25 min at 4°C and 250 L of supernatant was

transferred to a new tube and diluted to 1:10 in deionized water. Extracts were filtered with Ag filters (to eliminate Cl^-) and H^+ filters (to eliminate cations). Organic acid content was determined by ion chromatography in a Dionex DX-500 IC System (Thermo Fisher Scientific Inc., Waltham, MA, USA), that included a GP40 Gradient Pump and an ED40 Electrochemical Detector. For the separation Ion-Pak AG11 and AS11 columns were used and the gradient went from 0.2 mM NaOH to 15 mM NaOH in 25 min, at a flux of 1 mL min^{-1}.

1. 3. 10. Statistical analysis

a) *Pisum sativum* (PART I)

The data obtained from this study were analysed by the IBM SPSS Statistics (v.22). The mean was used as a measure of central tendency and the standard error (SE) as a measure of dispersion.

First, the data of the three different groups (No-Hypoxia, Hypoxia-24 and Hypoxia-48) were compared independently. The herbicide-treated plants were compared with their respective controls by the Student's *t*-Test for the Significance of the Difference between the Means of Two Independent Samples. A study along the time was not done, instead, for each sampling day, the data of the herbicide-treated and non-treated plants were compared in each of the groups. In all cases, statistical analyses were conducted at a significance level of 5% ($p < 0.05$).

Second, for all the studied parameters, a two-way analysis of variance (ANOVA) was done to examine the influence of the studied variables (hypoxia and herbicide application) and their possible interaction. These data are presented in tables. The statistical analyses was conducted at a significance level of 5% ($p < 0.05$).

b) *Arabidopsis thaliana* (PART II)

The data obtained from this study were analysed by the IBM SPSS Statistics (v.22). The mean was used as a measure of central tendency and the SE as a measure of dispersion.

First, for each studied parameter, the untreated plants of the two studied genotypes were compared by the Student's *t*-Test for the Significance of the Difference between the Means of Two Independent Samples. In all cases, statistical analyses were conducted at a significance level of 5% ($p < 0.05$).

Second, the data of the herbicide-treated and non-treated plants of each genotype were compared using the one-way ANOVA, after log transformations of the data if needed. In order to confirm homoscedasticity of variances, the Levene test was used. The HSD Tukey and Dunnett T3 *post hoc* statistical tests were applied to the homogeneity and non-homogeneity of variances cases, respectively. When the results were expressed in percentages, the data were previously transformed according to the following formula: $arcsin\sqrt{x/100}$. In all cases, statistical analyses were conducted at a significance level of 5% ($p < 0.05$).

1. 4. RESULTS AND DISCUSSION

1. 4. I. PART I: PHYSIOLOGICAL EFFECTS OF ABIHs IN PREHYPOXIC PEA PLANTS

In this section, the results obtained in the first experimental approach are presented and discussed. In this part, pea plants were exposed to low-oxygen conditions before the herbicide was applied, thus when the plants were treated with the herbicide they presented an enhanced fermentative metabolism. This way, the effects of the herbicide could be compared between plants showing an induced fermentative metabolism and others that did not have the fermentation pathway activated when the herbicide was applied.

For all the parameters studied, the results are presented in figures as follows: First, the results comparing the herbicide-treated and non-treated plants are presented for each specific group. Second, the results obtained in the two-way ANOVA (effects of the fixed factors (herbicide and hypoxia) and their interaction) are presented. And finally, the data of the IMX treated plants of each group with respect with respect to their controls (in percentages) are shown. The results obtained in the two-way ANOVA are presented in the Appendix A.

1. 4. I. 1. RESULTS (PART I)

a) Induction of ethanol fermentation

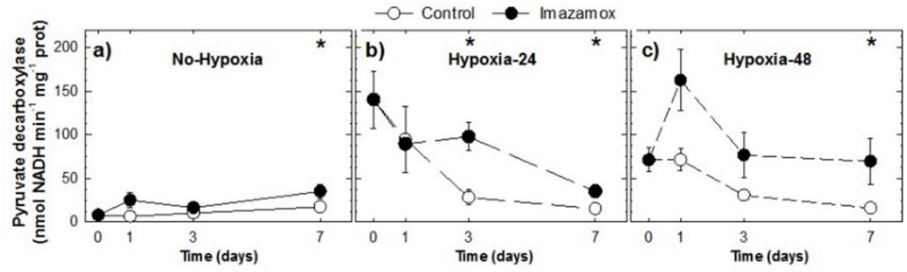
The *in vitro* enzymatic activities of PDC (Figure 1.6) and ADH (Figure 1.7) were measured in the roots of the plants from the studied six groups.

The PDC activity in the roots of the C-0 plants was about 10 nmol NADH min^{-1} mg^{-1} did not vary during the time course of the experiment (Figure 1.6.A.a). The herbicide application increased the activity of PDC in the roots of the plants of the No-Hypoxia group. At day 0, the PDC activity detected in the roots of the plants that were exposed to hypoxia (Figure 1.6.A.b and c) was much higher than the activity present in the roots of the plants from the No-Hypoxia group. Once the plants were again aerated, the PDC activity decreased to control values in the plants that were not treated with IMX. As it occurred in the No-Hypoxia group, PDC activity increased in IMX-treated plants from the groups Hypoxia-24 and Hypoxia-48.

According to the two-way ANOVA analysis (Figure 1.6.B), IMX had an effect on PDC activity the day 7 after herbicide application while Hypoxia had an effect on this parameter at day 1 after herbicide application. By contrast, Hypoxia did not affect PDC activity levels. No interaction was detected between the two fixed-factors.

Regarding the PDC activity of the IMX treated plants with respect to their respective controls (Figure 1.6.C), at the beginning of the experiment the plants that were not exposed to hypoxia presented higher values, however at the end of the experiment the PDC activity of the different groups represented as percentage of their controls was similar in the three groups.

A.



B.

	Day 1	Day 3	Day 7
Herbicide	0.169	0.988	**0.017**
Hypoxia	**0.003**	0.333	0.450
Interaction	0.161	0.051	0.412

C.

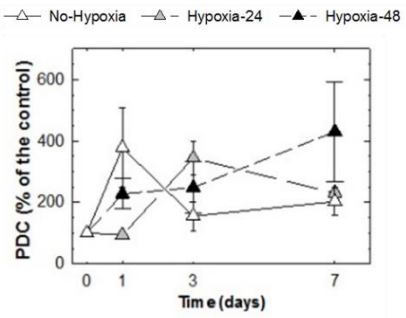
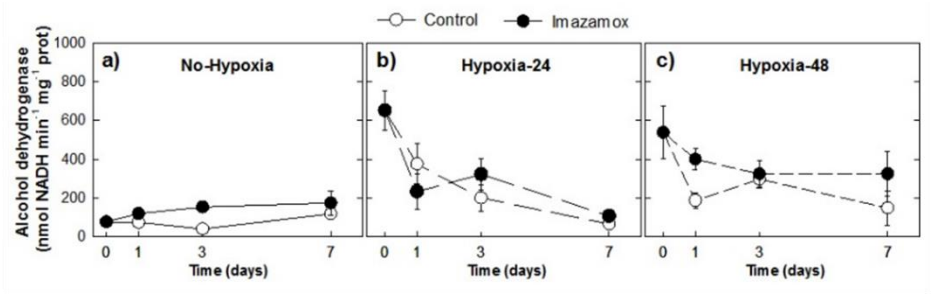


Figure 1.6. The effect of imazamox and hypoxia on the *in vitro* activity of pyruvate decarboxylase (PDC) in the roots of pea plants. Pea plants were not treated with hypoxia before herbicide application (Group No-Hypoxia) or were treated with hypoxia for 24 h before herbicide application (Group Hypoxia-24) or for 48 h (Group Hypoxia-48). Half of the plants from each group were treated with imazamox (5 mg active ingredient L^{-1}) and the other halves were not treated with herbicide and were the controls for the imazamox-treated plants of their group. **A.** Represents the *in vitro* activity of PDC in the roots of pea plants from the three studied groups, the days 0, 1, 3 and 7 after imazamox application. Values represent the mean ± SE (n = 4 biological replicates). Significant variations are marked with ★ for differences between control and imazamox-treated plants (*t*-Test, $p < 0.05$) at a given day. **B.** Two-way analysis of variance (ANOVA) indicating the influence of hypoxia and herbicide application on PDC activity and their possible interaction ($p < 0.05$). **C.** PDC activity of imazamox-treated plants with respect to the controls of each group. The original data are shown in section A.

A.



B.

	Day 1	Day 3	Day 7
Herbicide	0.632	0.682	0.205
Hypoxia	**0.001**	0.146	0.451
Interaction	0.081	0.757	0.598

C.

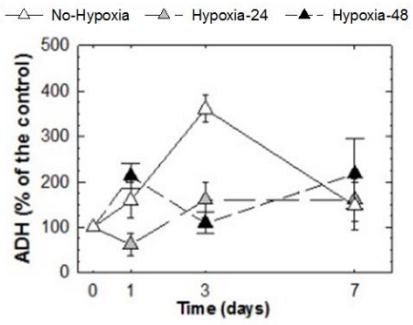


Figure 1.7. The effect of imazamox and hypoxia on the *in vitro* activity of alcohol dehydrogenase (ADH) in the roots of pea plants. Pea plants were not treated with hypoxia before herbicide application (Group No-Hypoxia) or were treated with hypoxia for 24 h before herbicide application (Group Hypoxia-24) or for 48 h (Group Hypoxia-48). Half of the plants from each group were treated with imazamox (5 mg active ingredient L^{-1}) and the other halves were not treated with herbicide and were the controls for the imazamox-treated plants of their group. **A.** Represents the *in vitro* activity of ADH in the roots of pea plants from the three studied groups, the days 0, 1, 3 and 7 after imazamox application. Values represent the mean ± SE (n = 4 biological replicates). **B.** Two-way analysis of variance (ANOVA) indicating the influence of hypoxia and herbicide application on ADH activity and their possible interaction ($p < 0.05$). **C.** ADH activity of imazamox-treated plants with respect to the controls of each group. The original data are shown in section A.

The *in vitro* activity of ADH in the roots of the group No-Hypoxia was increased as a consequence of IMX application (Figure 1.7.A.a). Regarding the groups that were exposed to hypoxia before herbicide application (Figure 1.7.A.b and c), as it occurred for PDC activity, at the beginning of the experiment the ADH activity was much higher comparing to the ADH activity of the group No-Hypoxia. Once the plants were again aerated, the activity of

ADH decreased to control values. The herbicide effect in the groups Hypoxia-24 and Hypoxia-48 was not very pronounced.

The two-way ANOVA analysis (Figure 1.7.B) indicates that IMX did not have an effect on ADH activity. However, the fixed factor Hypoxia affected ADH activity at day 1. No interaction was detected between the two fixed-factors.

Regarding the ADH activity of the IMX-treated plants with respect to their controls (Figure 1.7.C), no clear pattern was observed.

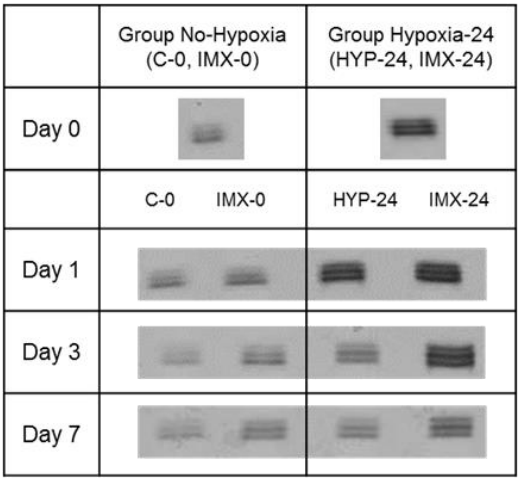


Figure 1.8. Native PAGE of root ADH activity 0, 1, 3 and 7 days after herbicide application. Pea plants were not treated with hypoxia before herbicide application (Group No-Hypoxia) or were treated with hypoxia for 24 h before herbicide application (Group Hypoxia-24). Half of the plants from each group were treated with imazamox (5 mg active ingredient L^{-1}) and were named IMX-0 (group No-hypoxia) and IMX-24 (group Hypoxia-24). The other halves were not treated with herbicide and were the controls for the imazamox-treated plants. They were named C-0 (group No-hypoxia) and HYP-24 (group Hypoxia-24). Each lane contained 1.75 μg protein.

The enzymatic activity of ADH was determined by native electrophoresis in the roots of the plants from groups No-Hypoxia and Hypoxia-24 (Figure 1.8). The three ADH isoenzymes described in pea were detected (from top to bottom: ADH1-ADH1, ADH1-ADH2 and ADH2-ADH2).

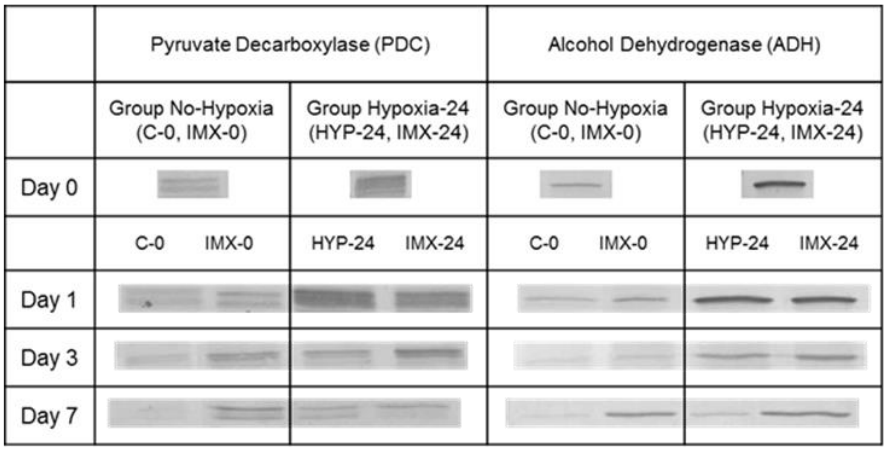
At day 0, the activity of three ADH isoenzymes of the plants from the group Hypoxia-24 was higher than the activity of the plants from the group No-Hypoxia. In the plants HYP-24 (non-treated with IMX) the ADH activity decreased in the following days.

In the group No-Hypoxia, IMX application increased the activity of the ADH1-ADH2 and ADH2-ADH2 isoenzymes from day 3. The activity of ADH in the plants IMX-24 was much higher than the activity of IMX-0 (non-treated with hypoxia) during all the course of the experiment.

The protein content of the enzymes involved in ethanol fermentation was analysed in the plants from groups No-Hypoxia and Hypoxia-24 (Figure 1.9). The intensity of the bands was also measured and is presented in Figure 1.9.A. Similar pattern was observed regarding the PDC and ADH protein content in both groups. At day 0, both PDC and ADH protein content was much higher than in the group Hypoxia-24 comparing to the group No-Hypoxia. In the plants non-treated with IMX from group Hypoxia-24 (HYP-24) the PDC and ADH content decreased along the experiment and even reached control (C-0) levels at the end of the studied period (day 7).

In the group No-Hypoxia, both PDC and ADH protein contents increased as a consequence of herbicide application. By contrast, PDC content of IMX-treated plants from group No-hypoxia decreased and at day 7 it reached control values, however, the ADH content also decreased but it maintained higher than the levels of its controls at the end of the experiment.

A.



B.

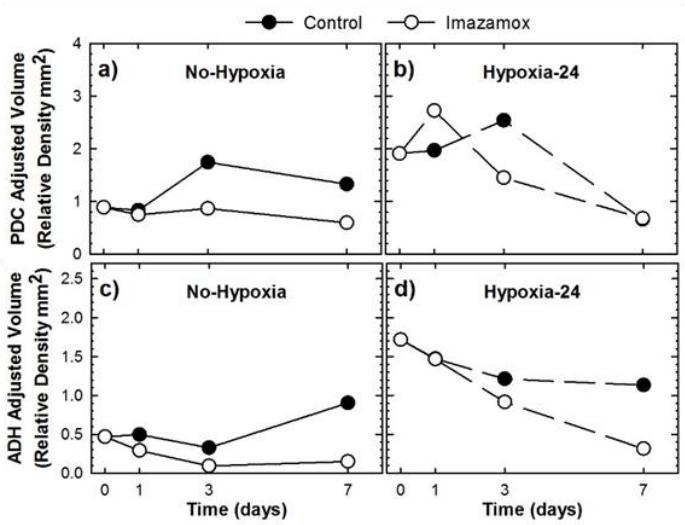
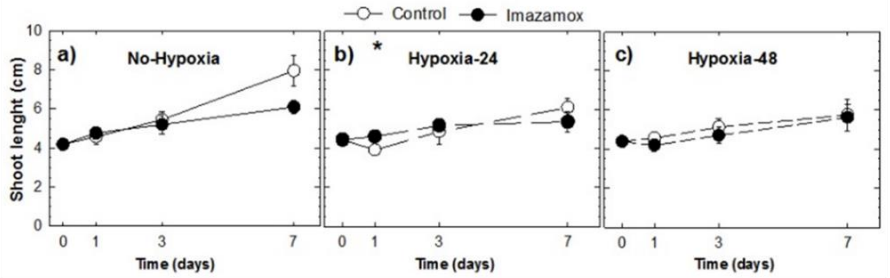


Figure 1.9 Pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) protein contents in the roots of pea plants. Pea plants were not treated with hypoxia before herbicide application (Group No-Hypoxia) or were treated with hypoxia for 24 h before herbicide application (Group Hypoxia-24). Half of the plants from each group were treated with imazamox (5 mg active ingredient L^{-1}) and were named IMX-0 (group No-hypoxia) and IMX-24 (group Hypoxia-24). The other halves were not treated with herbicide and were the controls for the imazamox-treated plants. They were named C-0 (group No-hypoxia) and HYP-24 (group Hypoxia-24). **A.** Immunoblot detection of PDC and ADH at days 0, 1, 3 and 7 after imazamox application. For each treatment, one representative sample is present. Each lane contained 30 µg protein. **B.** Representation of the intensity of the bands from the immunoblot detection of PDC and ADH at days 0, 1, 3 and 7 days after herbicide application. Values represent the mean ± SE (n = 4 biological replicates).

b) Growth arrest

In order to study the effect of IMX on the growth of the different studied groups, the shoot (Figure 1.10) and root (Figure 1.11) lengths were measured.

A.



B.

	Day 1	Day 3	Day 7
Herbicide	0.515	0.738	0.087
Hypoxia	0.082	0.631	0.066
Interaction	0.144	0.681	0.392

C.

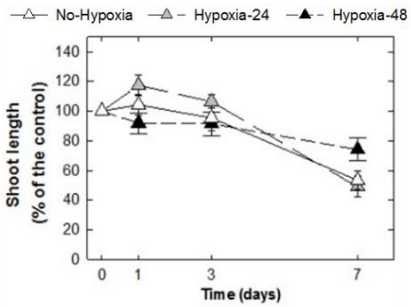


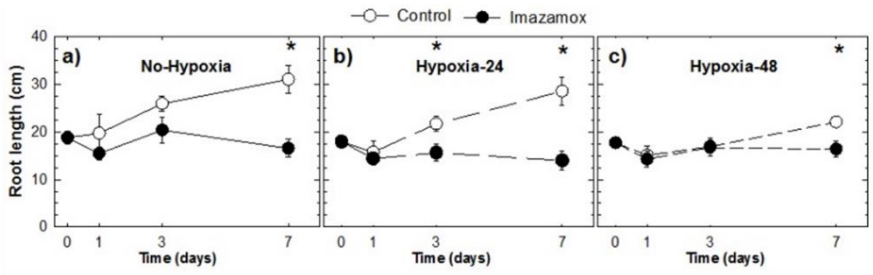
Figure 1.10. The effect of imazamox and hypoxia on the shoot growth of pea plants. Pea plants were not treated with hypoxia before herbicide application (Group No-Hypoxia) or were treated with hypoxia for 24 h before herbicide application (Group Hypoxia-24) or for 48 h (Group Hypoxia-48). Half of the plants from each group were treated with imazamox (5 mg active ingredient L^{-1}) and the other halves were not treated with herbicide and were the controls for the imazamox-treated plants of their group. **A.** Represents the shoot length of pea plants from the three studied groups, the days 0, 1, 3 and 7 after imazamox application. Values represent the mean ± SE (n = 4 biological replicates). Significant variations are marked with * for differences between control and imazamox-treated plants (*t*-Test, $p < 0.05$) at a given day. **B.** Two-way analysis of variance (ANOVA) indicating the influence of hypoxia and herbicide application on the shoot length and their possible interaction ($p < 0.05$). **C.** The shoot length of imazamox-treated plants with respect to the controls of each group. The original data are shown in section A.

The shoot of the plants from the different groups grew in a similar way during the experiment, however, in the group No-Hypoxia a decrease in the shoot length was observed as a consequence of IMX application after 7 days of treatment (Figure 1.10.A.a), although the differences were not statistically

significant. This difference was alleviated in both groups exposed to hypoxia where no differences in the shoot length between HYP-24 and IMX-24 (Figure 1.10.A.b) and between HYP-48 and IMX-48 (Figure 1.10.A.c) were detected.

The two-way ANOVA analysis showed that the fixed factors had no effect on the shoot growth and that no interaction was present between both fixed factors (Herbicide and Hypoxia) (Figure 1.10.B).

A.



B.

	Day 1	Day 3	Day 7
Herbicide	0.531	**0.014**	**0.000**
Hypoxia	**0.012**	**0.006**	0.064
Interaction	0.963	0.229	0.219

C.

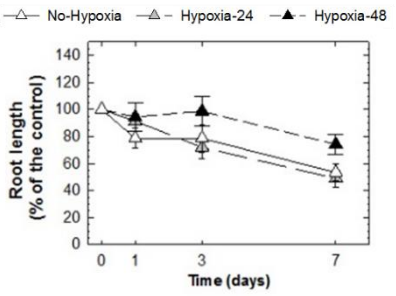


Figure 1.11. The effect of imazamox and hypoxia on the root growth of pea plants. Pea plants were not treated with hypoxia before herbicide application (Group No-Hypoxia) or were treated with hypoxia for 24 h before herbicide application (Group Hypoxia-24) or for 48 h (Group Hypoxia-48). Half of the plants from each group were treated with imazamox (5 mg active ingredient L^{-1}) and the other halves were not treated with herbicide and were the controls for the imazamox-treated plants of their group. **A.** Represents the root length of pea plants from the three studied groups, the days 0, 1, 3 and 7 after imazamox application. Values represent the mean ± SE (n = 4 biological replicates). Significant variations are marked with * for differences between control and imazamox-treated plants (*t*-Test, $p < 0.05$) at a given day. **B.** Two-way analysis of variance (ANOVA) indicating the influence of hypoxia and herbicide application on the root length and their possible interaction ($p < 0.05$). **C.** The root length of imazamox-treated plants with respect to the controls of each group. The original data are shown in section A.

When representing the shoot length of the IMX-treated plants of the different groups with respect to each control (Figure 1.10.C), no differences were detected. At day 7, the plants IMX-48 were less affected by the herbicide than the plants from the other two groups.

A decrease in the root length was observed in all the groups as a consequence of herbicide application after 7 days of treatment and in the Hypoxia-24 group also after 3 days after herbicide application (Figure 1.11.A).

The two-way ANOVA analysis (Figure 1.11.B) showed herbicide effect after 3 and 7 days of treatment and Hypoxia effect after 1 and 3 days of herbicide application. No interaction was detected between both fixed factors.

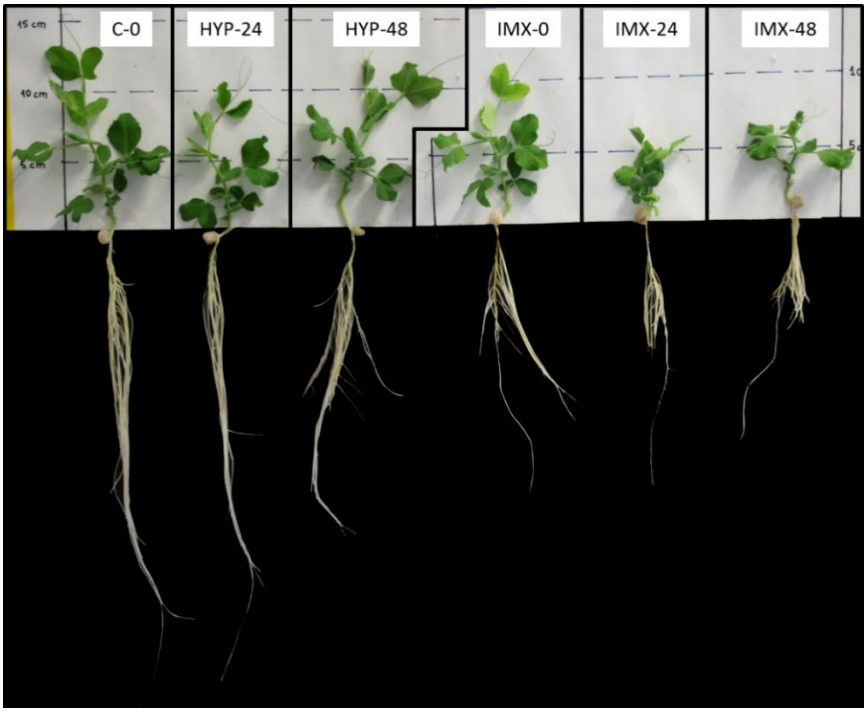


Figure 1.12. Pea plants non-treated or treated with imazamox for seven days. C-0: non-treated control plants; HYP-24 and HYP-48: plants non-treated with imazamox and exposed to hypoxia for 24 and 48 hours, respectively; IMX-0: imazamox-treated plants and not exposed to hypoxia; IMX-24 and IMX-48: imazamox-treated plants and exposed to hypoxia for 24 or 48 hours, respectively, before herbicide application.

When representing the root length of the IMX-treated plants of the different groups respect to each control (Figure 1.11.C), it was seen that the plants from the IMX-48 treatment had less differences with their controls (HYP-48) than the differences observed between the herbicide-treated and non-treated plants from the studied other two groups.

Figure 1.12 shows the aspect of the plants seven days after herbicide application. The roots of the IMX-treated plants became brownish while the roots of the plants non-treated with IMX was white. The growth of the secondary roots was inhibited and the growth of the shoot and the principal root was also arrested.

c) Total Free Amino Acid Accumulation

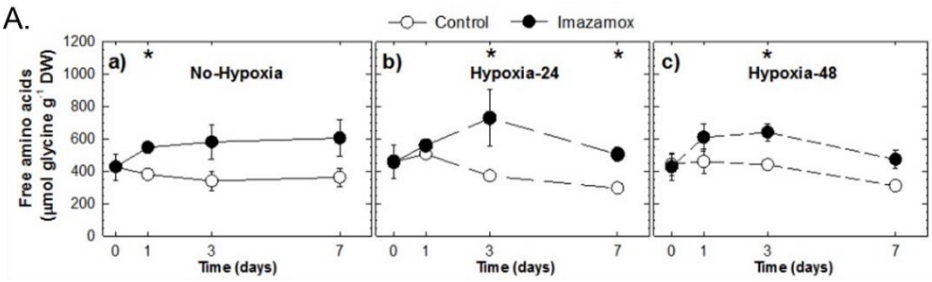
An increase in the total free amino acid content has been described as a consequence of AHAS inhibition in different plant species (Shaner and Reider, 1986; Zabalza et al., 2006; García-Garijo et al., 2012; Zabalza et al., 2013).

In the roots of the plants from all the studied groups higher levels of total free amino acids were observed as a consequence of IMX application (Figure 1.13.A).

In the two-ways ANOVA the fixed factor herbicide had an effect on the total free amino acid levels after 3 days from the herbicide application, instead, Hypoxia did not affect this parameter (Figure 1.13.B).

Regarding the % of the herbicide treated plants in each group with respect to their controls (Figure 1.13.C), no differences between the three groups were detected.

A.



B.

	Day 1	Day 3	Day 7
Herbicide	0.064	**0.001**	**0.001**
Hypoxia	0.522	0.457	0.273
Interaction	0.746	0.607	0.823

C.

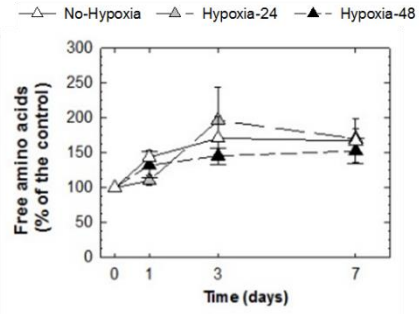
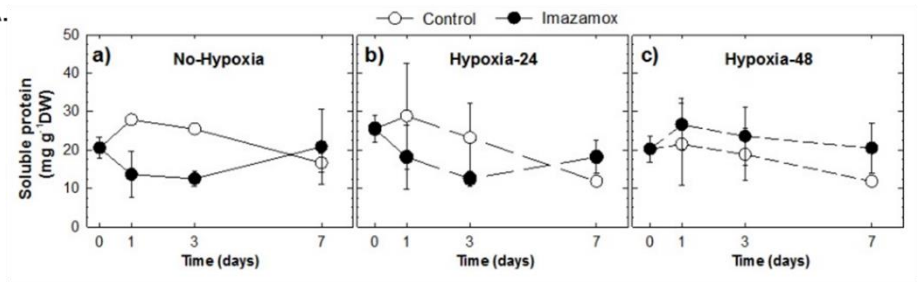


Figure 1.13. The effect of imazamox and hypoxia on the total free amino acid content in the root of pea plants. Pea plants were not treated with hypoxia before herbicide application (Group No-Hypoxia) or were treated with hypoxia for 24 h before herbicide application (Group Hypoxia-24) or for 48 h (Group Hypoxia-48). Half of the plants from each group were treated with imazamox (5 mg active ingredient L^{-1}) and the other halves were not treated with herbicide and were the controls for the imazamox-treated plants of their group. **A.** Represents the content of free amino acids in the roots of pea plants from the three studied groups, the days 0, 1, 3 and 7 after imazamox application. Values represent the mean ± SE (n = 4 biological replicates). Significant variations are marked with ★ for differences between control and imazamox-treated plants (*t*-Test, $p < 0.05$) at a given day. **B.** Two-way analysis of variance (ANOVA) indicating the influence of hypoxia and herbicide application on the total free amino acid content and their possible interaction ($p < 0.05$). **C.** The free amino acid content of imazamox-treated plants with respect to the controls of each group. The original data are shown in section A.

d) Effects in the soluble protein content

A.



B.

	Day 1	Day 3	Day 7
Herbicide	0.238	0.455	0.209
Hypoxia	0.910	0.959	0.797
Interaction	0.405	0.505	0.928

C.

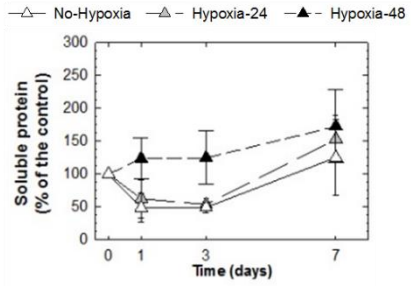


Figure 1.14. The effect of imazamox and hypoxia on the soluble protein content in the root of pea plants. Pea plants were not treated with hypoxia before herbicide application (Group No-Hypoxia) or were treated with hypoxia for 24 h before herbicide application (Group Hypoxia-24) or for 48 h (Group Hypoxia-48). Half of the plants from each group were treated with imazamox (5 mg active ingredient L^{-1}) and the other halves were not treated with herbicide and were the controls for the imazamox-treated plants of their group. **A.** Represents the soluble protein content in the roots of pea plants from the three studied groups, the days 0, 1, 3 and 7 after imazamox application. Values represent the mean ± SE (n = 4 biological replicates). Significant variations are marked with ★ for differences between control and imazamox-treated plants (*t*-Test, $p < 0.05$) at a given day. **B.** Two-way analysis of variance (ANOVA) indicating the influence of hypoxia and herbicide application on the soluble protein content and their possible interaction ($p < 0.05$). **C.** The soluble protein content of imazamox-treated plants with respect to the controls of each group. The original data are shown in section A.

A decrease in the soluble protein has been described as a consequence of AHAS inhibition (Wittenbach and Abell, 1999; Gaston et al., 2002; Zulet et al., 2013a). In this experiment IMX provoked a decrease in the soluble protein levels in the No-Hypoxia (Figure 1.14.A.a) and although no significant differences were found between HYP-24 and IMX-24 (probably due to the big SEs) the soluble protein content of IMX-24 plants were lower than their control (Figure

1.14.A.b). On the contrary, the soluble protein content increased in the IMX-treated plants from the group Hypoxia-48 (Figure 1.14.A.c).

No herbicide and Hypoxia effect on the soluble protein content was observed in the two-way ANOVA analysis, and no interaction was detected between the two analysed fixed factors (Figure 1.14.B).

Regarding the % of soluble protein content of the IMX treated-plants with respect to their controls (Figure 1.14.C), IMX-0 and IMX-24 presented similar decrease comparing to the non-herbicide treated controls, while the protein content of IMX-48 was higher comparing to the control.

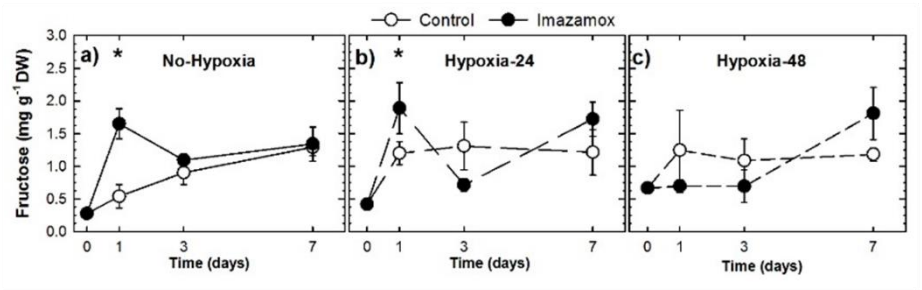
e) Increase in the carbohydrate content

An increase in the carbohydrate content been described in plants as a consequence of AHAS inhibition (Gaston et al., 2002; Zabalza et al., 2004). Fructose (Figure 1.15), glucose (Figure 1.16), sucrose (Figure 1.17), their sum (Figure 1.18) and starch (Figure 1.19) contents were measured in the roots of the pea plants.

An accumulation of fructose was observed in the roots of IMX-treated plants one day after herbicide application in the plants from the groups No-Hypoxia and Hypoxia-24 (Figure 1.15.A). Later, no differences in the fructose content in any of the three studied groups was detected as a consequence of IMX application.

An effect of both fixed factors was detected on the fructose content one day after herbicide application, they also presented significant interaction, indicating that the effect of IMX depended on the presence of Hypoxia at day 1 (Figure 1.15.B).

A.



B.

	Day 1	Day 3	Day 7
Herbicide	**0.001**	0.248	0.102
Hypoxia	**0.002**	0.873	0.782
Interaction	**0.002**	0.375	0.552

C.

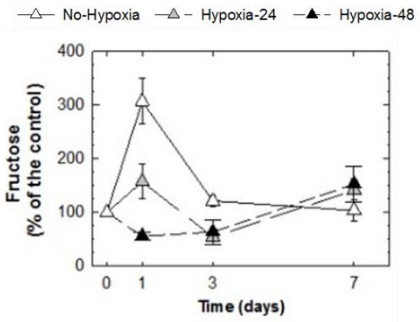
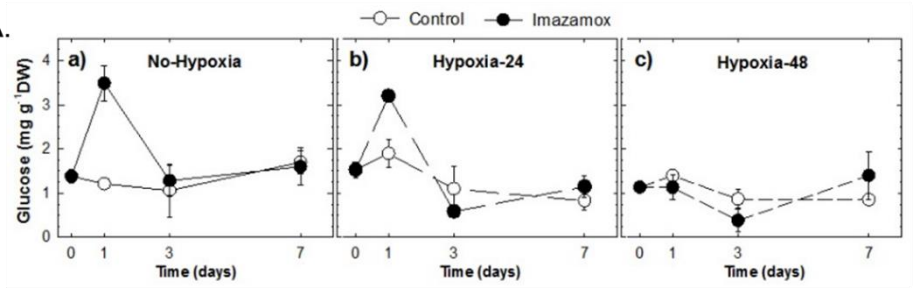


Figure 1.15. The effect of imazamox and hypoxia on the fructose content in the root of pea plants. Pea plants were not treated with hypoxia before herbicide application (Group No-Hypoxia) or were treated with hypoxia for 24 h before herbicide application (Group Hypoxia-24) or for 48 h (Group Hypoxia-48). Half of the plants from each group were treated with imazamox (5 mg active ingredient L^{-1}) and the other halves were not treated with herbicide and were the controls for the imazamox-treated plants of their group. **A.** Represents the fructose content in the roots of pea plants from the three studied groups, the days 0, 1, 3 and 7 after imazamox application. Values represent the mean ± SE (n = 4 biological replicates). Significant variations are marked with * for differences between control and imazamox-treated plants (*t*-Test, $p < 0.05$) at a given day. **B.** Two-way analysis of variance (ANOVA) indicating the influence of hypoxia and herbicide application on the fructose content and their possible interaction ($p < 0.05$). **C.** The fructose content of imazamox-treated plants with respect to the controls of each group. The original data are shown in section A.

When representing the fructose content of the herbicide-treated plants with respect to each control (Figure 1.15.C) an alleviation in the fructose accumulation was detected at day 1 and slightly at day 3 in the hypoxia treated plants, with respect to the plants that were not exposed to hypoxia conditions before herbicide application.

A.



B.

	Day 1	Day 3	Day 7
Herbicide	**0.001**	0.384	0.351
Hypoxia	**0.002**	0.341	0.143
Interaction	**0.002**	0.539	0.621

C.

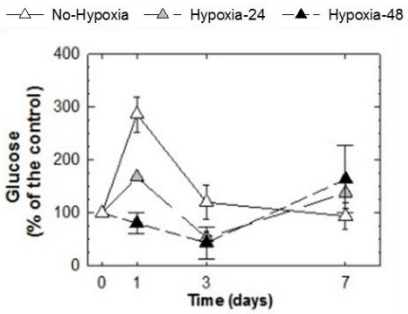


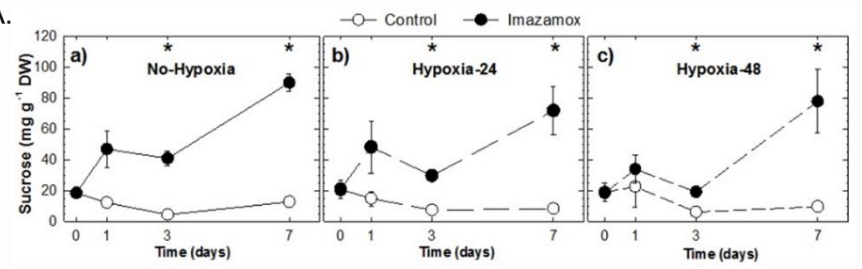
Figure 1.16. The effect of imazamox and hypoxia on the glucose content in the root of pea plants. Pea plants were not treated with hypoxia before herbicide application (Group No-Hypoxia) or were treated with hypoxia for 24 h before herbicide application (Group Hypoxia-24) or for 48 h (Group Hypoxia-48). Half of the plants from each group were treated with imazamox (5 mg active ingredient L^{-1}) and the other halves were not treated with herbicide and were the controls for the imazamox-treated plants of their group. **A.** Represents the glucose content in the roots of pea plants from the three studied groups, the days 0, 1, 3 and 7 after imazamox application. Values represent the mean ± SE (n = 4 biological replicates). Significant variations are marked with * for differences between control and imazamox-treated plants (*t*-Test, $p < 0.05$) at a given day. **B.** Two-way analysis of variance (ANOVA) indicating the influence of hypoxia and herbicide application on the glucose content and their possible interaction ($p < 0.05$). **C.** The glucose content of imazamox-treated plants with respect to the controls of each group. The original data are shown in section A.

An accumulation of glucose was observed in the roots of IMX-treated plants one day after herbicide application in the plants from the groups No-Hypoxia (Figure 1.16.A.a) and Hypoxia-24 (Figure 1.16.A.b). Later, the glucose content decreased and it was similar to the values in their respective control plants. In the group Hypoxia-48, both treatments presented similar glucose content during all the experiment (Figure 1.16.A.c).

An effect of both fixed factors was detected on the glucose content the day 1 after herbicide application, they also presented significant interaction, indicating that the effect of IMX depended on the presence of Hypoxia at day 1 (Figure 1.16.B).

When representing the glucose content of the herbicide-treated plants with respect to each control (Figure 1.16.C) an alleviation in the glucose accumulation was detected at day 3 and slightly at day 1 in the hypoxia treated plants, with respect to the plants that were not exposed to hypoxia conditions before herbicide application.

A.



B.

	Day 1	Day 3	Day 7
Herbicide	**0.020**	**0.000**	**0.000**
Hypoxia	0.756	**0.013**	0.337
Interaction	0.612	**0.004**	0.659

C.

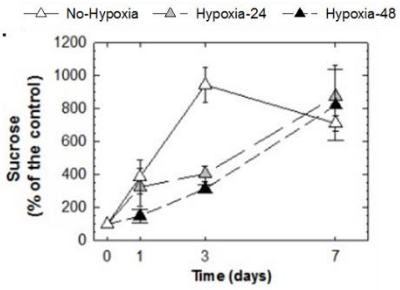


Figure 1.17. The effect of imazamox and hypoxia on the sucrose content in the root of pea plants. Pea plants were not treated with hypoxia before herbicide application (Group No-Hypoxia) or were treated with hypoxia for 24 h before herbicide application (Group Hypoxia-24) or for 48 h (Group Hypoxia-48). Half of the plants from each group were treated with imazamox (5 mg active ingredient L^{-1}) and the other halves were not treated with herbicide and were the controls for the imazamox-treated plants of their group. **A.** Represents the sucrose content in the roots of pea plants from the three studied groups, the days 0, 1, 3 and 7 after imazamox application. Values represent the mean ± SE (n = 4 biological replicates). Significant variations are marked with * for differences between control and imazamox-treated plants (*t*-Test, $p < 0.05$) at a given day. **B.** Two-way analysis of variance (ANOVA) indicating the influence of hypoxia and herbicide application on the sucrose content and their possible interaction ($p < 0.05$). **C.** The sucrose content of imazamox-treated plants with respect to the controls of each group. The original data are shown in section A.

An increase in the sucrose content was detected in the IMX treated plants of the three studied groups, from the day 3 after herbicide application (Figure 1.17.A.a).

The two-way ANOVA analysis indicated an effect of the herbicide during all the experiment and an effect of Hypoxia the day 3 after herbicide application, an interaction of both fixed factors was also observed at day 3 (Figure 1.17.B).

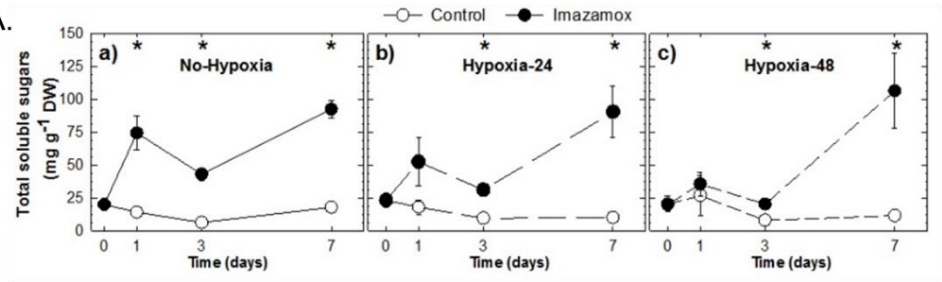
At day 3, an alleviation of the sucrose accumulation provoked by IMX was observed in the plants that were exposed to hypoxia before herbicide application, but at day 7 all the groups presented similar values (Figure 1.17.C).

Since sucrose is the most abundant soluble carbohydrate in the roots, similar effects of the herbicide were detected in the total soluble carbohydrate content to the ones found in sucrose content. Thus, total carbohydrates were accumulated in the herbicide-treated plants from all the studied groups (Figure 1.18.A).

The herbicide had an effect on the total soluble carbohydrate content during all the experiment, Hypoxia effect was significant the day 3 after herbicide application. At this day, an interaction of both fixed factors was detected (Figure 1.18.B).

The accumulation of total soluble sugars observed in the IMX-0 plants was calculated and presented as percentage with respect to their controls, it was alleviated in the hypoxia treated plants the days 1 and 3 after herbicide treatment (Figure 1.18.C).

A.



B.

	Day 1	Day 3	Day 7
Herbicide	**0.001**	**0.000**	**0.000**
Hypoxia	0.464	**0.018**	0.809
Interaction	0.078	**0.005**	0.761

C.

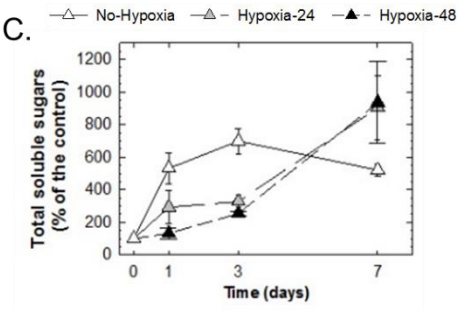
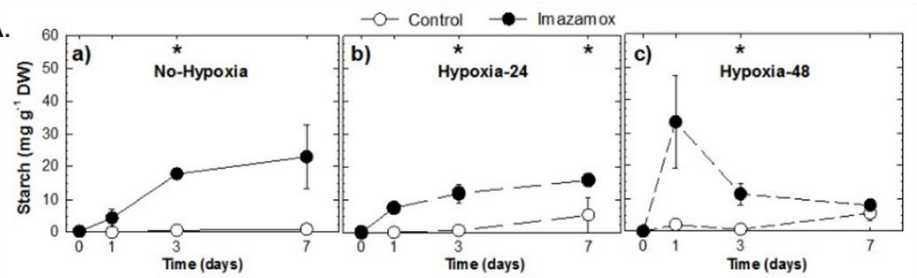


Figure 1.18. The effect of imazamox and hypoxia on the total soluble sugar content in the root of pea plants. Pea plants were not treated with hypoxia before herbicide application (Group No-Hypoxia) or were treated with hypoxia for 24 h before herbicide application (Group Hypoxia-24) or for 48 h (Group Hypoxia-48). Half of the plants from each group were treated with imazamox (5 mg active ingredient L^{-1}) and the other halves were not treated with herbicide and were the controls for the imazamox-treated plants of their group. **A.** Represents the total soluble sugar content in the roots of pea plants from the three studied groups, the days 0, 1, 3 and 7 after imazamox application. Values represent the mean ± SE (n = 4 biological replicates). Significant variations are marked with * for differences between control and imazamox-treated plants (*t*-Test, $p < 0.05$) at a given day. **B.** Two-way analysis of variance (ANOVA) indicating the influence of hypoxia and herbicide application on the total soluble sugar content and their possible interaction ($p < 0.05$). **C.** The total soluble sugar content of imazamox-treated plants with respect to the controls of each group. The original data are shown in section A.

Starch was also accumulated in the roots of the plants of the three studied groups as a consequence of IMX application (Figure 1.19.A).

According to the two-way ANOVA analysis (Figure 1.19.B) the Herbicide had an effect on the starch content during all the time course of the experiment. By contrast, Hypoxia did not affect this parameter. No interaction was detected between both fixed factors.

A.



B.

	Day 1	Day 3	Day 7
Herbicide	**0.034**	**0.000**	**0.008**
Hypoxia	0.151	0.996	0.364
Interaction	0.193	0.625	0.080

C.

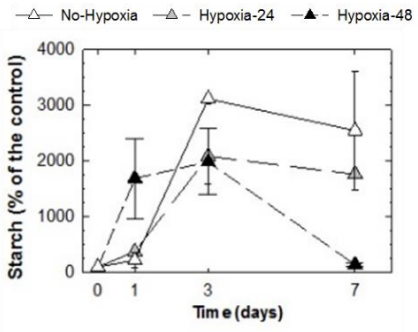


Figure 1.19. The effect of imazamox and hypoxia on the starch content in the root of pea plants. Pea plants were not treated with hypoxia before herbicide application (Group No-Hypoxia) or were treated with hypoxia for 24 h before herbicide application (Group Hypoxia-24) or for 48 h (Group Hypoxia-48). Half of the plants from each group were treated with imazamox (5 mg active ingredient L^{-1}) and the other halves were not treated with herbicide and were the controls for the imazamox-treated plants of their group. **A.** Represents the starch content in the roots of pea plants from the three studied groups, the days 0, 1, 3 and 7 after imazamox application. Values represent the mean ± SE (n = 4 biological replicates). Significant variations are marked with * for differences between control and imazamox-treated plants (*t*-Test, $p < 0.05$) at a given day. **B.** Two-way analysis of variance (ANOVA) indicating the influence of hypoxia and herbicide application on the starch content and their possible interaction ($p < 0.05$). **C.** The starch content of imazamox-treated plants with respect to their controls of each group. The original data are shown in section A.

In the relative representation of each group for the herbicide effect in % respect to control values the plants exposed to hypoxia before herbicide application showed lower starch accumulation at days 3 and 7 than the plants that were not exposed to hypoxia (Figure 1.19.C).

1. 4. I. 2. DISCUSSION (PART I)

a) Validation of the experiment / desired conditions

The oxygen concentration in the nutrient solution measurements and the *in vitro* activities of the enzymes involved in the ethanol fermentation validated the experimental design since the desired conditions were obtained. The oxygen concentration in the nutrient solution of the plants exposed to hypoxia before herbicide application drastically decreased and it was about 30-40% the day of herbicide application (day 0). On the other hand, at day 0, the activities of PDC and ADH in the roots of the plants from the groups Hypoxia-24 and Hypoxia-48 was much higher than the activities of the No-Hypoxia plants. Thus, the ethanol fermentation in the roots was already induced when the herbicide was applied.

b) Typical response to AHAS inhibition was observed

The already described effects triggered as a consequence of AHAS inhibition were observed in the IMX treated plants.

An induction in the ethanol fermentation was observed as a consequence of IMX application since the *in vitro* activities of both PDC and ADH increased (Figure 1.6 and 1.7). Moreover, IMX also provoked an increase in the in gel ADH activity (Figure 1.8) and the protein content of both PDC and ADH were higher in IMX-treated plants (Figure 1.9). Ethanol fermentation has been described to be induced in plants in response to several AHAS inhibitors. An induction of PDC and ADH in vitro activities has been observed in the roots of soybean and pea plants after AHAS inhibition, moreover, immunoblot analysis also demonstrated an accumulation of the corresponding proteins (Gaston et al., 2002; Zabalza et al., 2005; Zulet et al., 2013a).

A growth arrest has been described in several plant species (e.g. soybean, pea, rice) as a consequence of AHAS inhibition (Wittenbach and Abell, 1999; Gaston et al., 2003; Zabalza et al., 2007; Qian et al., 2009). The results obtained

in this experiment also shown that IMX provoked a decrease in both shoot and root elongation (Figure 1.10 and 1.11).

Moreover, regarding the nitrogen metabolism, an increase in the total free amino acid content (Figure 1.13) and a slight decrease in the soluble protein content (Figure 1.14) were observed in the roots of pea plants after IMX application. These response has been previously observed in plants treated with different AHAS inhibitors (Shaner and Reider, 1986; Zabalza et al., 2006; Zulet et al., 2013a) and it has been proposed to occur due to an increased protein turnover (Rhodes et al., 1987). The main proteolytic systems has been studied in the roots of ABIH-treated pea plants and it was observed that although they all were affected, the activity of all the proteolytic systems did not increase (Zulet et al., 2013a).

Carbohydrates were accumulated in the roots as a consequence of AHAS inhibition since an increase in the total soluble sugars (Figure 1.18) and starch content in roots (Figure 1.19) were observed. Regarding the individual soluble sugars, an accumulation of glucose (Figure 1.16) and the most abundant sugar, sucrose (Figure 1.17), were detected in the roots of IMX-treated pea plants.

Carbohydrate accumulation has also been described as a consequence of AHAS inhibition (Gaston et al., 2002; Zabalza et al., 2004; Qian et al., 2011b). Carbohydrate accumulation detected in sinks after AHAS inhibition has been observed to be followed by an accumulation in source organs (Zabalza et al., 2004). This has been explained to occur as follows: as growth is arrest, carbohydrates transported to the sinks are not metabolized and they accumulate. Since carbohydrates are transported at a higher rate than they are being transported from the leaves to the roots, the sugar gradient required for long-distance transport is abolished and phloem transport is inhibited. As a consequence, carbohydrate accumulation in the leaves of treated plants occurred because of a decrease in sink strength (Zabalza et al., 2004).

In general, after the target of an inhibitor has been affected, death occurs as a result of various causes. First, death could be provoked by starvation of the end products that are generated in the inhibited pathway. Second, an accumulation of the substrates of the inhibited target could result in lethality. And third, several reactions can be triggered after the inhibition of the target that could be associated with the plant death. As a consequence of AHAS inhibition no branched-chain amino acid limitation is detected in the treated plants, and thus, death does not occur due to end product starvation. Another effects that result from AHAS inhibition are the induction of fermentation and alternative oxidase pathway. Both pathways share pyruvate as common substrate with the inhibited target, and thus, it can be proposed that these pathways are induced to prevent pyruvate accumulation. Plant death following AHAS inhibition, has been associated with an impairment of carbon and nitrogen metabolism. Studies with two inhibitors of the branched-chain amino acid pathway at different steps, showed that although they had common effects on carbon metabolism, only the AHAS inhibitor induced an increase in the free amino acid content (Zabalza et al., 2013). Thus, the different herbicidal efficacy observed after the two targets inhibition could be associated with a different carbon/nitrogen metabolism imbalance (Zabalza et al., 2013).

c) Slight alleviation of the physiological consequences if fermentation is induced before herbicide application

The IMX-treated plants from the groups that were exposed to hypoxia before herbicide application also presented the typical response described in plants as a consequence of AHAS inhibition. However, the herbicide effect was slightly alleviated when plants were exposed to low-oxygen conditions for 48 h before herbicide application, indicating that having the ethanol fermentation activated 48 h before IMX application decreases the toxicity of the herbicide and suggest that fermentation induction is part of the plant defence mechanism after herbicide treatment.

The shoot and root growth arrest of the IMX-48 plants compared to their controls (HYP-48) was less severe than the growth arrest observed in the other studied groups.

As for the nitrogen metabolism markers, no changes in the total free amino acid profile were observed in the IMX-48 plants comparing to the other IMX-treated plants. However, contrary to what was observed for the IMX-treated plants from the groups No-Hypoxia and Hypoxia-24, the soluble protein content increased in the IMX-48 plants.

Regarding the carbohydrate content, although an accumulation of carbohydrates was detected in the roots of IMX-48 plants, the increase observed in these plants comparing to their controls was less pronounced than the accumulation found in the plants from the other studied groups.

The results obtained in the first experimental approach of this Chapter indicate that having the ethanol fermentation activated before IMX application slightly decreases the typical physiological effects of the herbicide and suggest that the induction of fermentation is part of the plant defence mechanism after the herbicide treatment.

1. 4. II. PART II: PHYSIOLOGICAL EFFECTS OF ABIHs IN *A. thaliana* PLANTS LACKING FERMENTATION

In the second experimental approach of this chapter, the effects ABIH in *A. thaliana* plants lacking the *ADH1* gene were compared with the effects provoked by ABIH on *A. thaliana* wild-plants. One inhibitor of the AHAS (IMX) and one inhibitor of the EPSPS (GPL) were used. This way, the importance of the ethanol fermentation in the response of the plants to ABIH application was evaluated.

1. 4. II. 1. RESULTS (PART II)

a) Dose determination

Preliminary studies were conducted to determine the IMX and GLP concentrations that induce similar effects to those previously described in pea plants (Zabalza et al., 2004; Zabalza et al., 2005; Orcaray et al., 2012; Zulet et al., 2013a). The herbicides concentrations used in these previous studies provoke pea death within 20 days (Orcaray et al., 2010).

Net carbon dioxide assimilation rates were measured from the youngest, fully expanded leaf and we found that 4.9 μM IMX and 87.65 μM GLP caused plant death at 20 days, when photosynthesis was almost zero, in the two genotypes evaluated (Figure 1.20).

b) Validation of the use of *A. thaliana* for the study of the physiological effects triggered after ABIH application

To determine whether *A. thaliana* was suitable for the study of the effects provoked by ABIH, the already described effects triggered after AHAS or EPSPS inhibition in plants were evaluated on this model plant (induction of fermentation and carbohydrates and free amino acids accumulation) (Zabalza et al., 2004; Zabalza et al., 2005; Orcaray et al., 2012; Zulet et al., 2013a). For that purpose, *A. thaliana* wild-type plants were treated with IMX or GLP (for three days) and the herbicide effects on the *in vitro* activity of the enzymes involved in the ethanol fermentation were measured in the roots and the total soluble

sugars content, the starch content and the total free amino acid content were monitored in the leaves and the roots of *A. thaliana* plants.

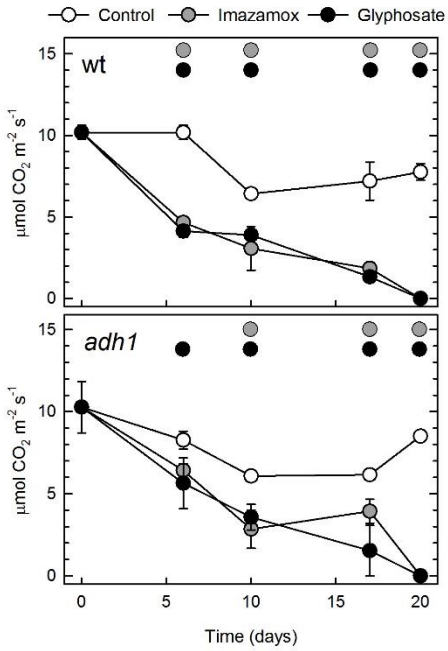


Figure 1.20. Net photosynthesis in the leaves of untreated *Arabidopsis thaliana* (wild-type (wt) Col-0 and *adh1* mutants) plants or plants treated with imazamox or glyphosate applied to the nutrient solution. Measurements were made on the youngest, fully-expanded leaf. Each value represents the mean ± SE (n = 10 - 15). ● and ● indicate significant differences between control and imazamox or glyphosate treated plants, respectively ($p < 0.05$), for a given day.

As a consequence of IMX or GLP application, an induction of the fermentative metabolism was observed in the roots of *A. thaliana* plants, since an increase in the activities of both PDC (Figure 1.21.A.a) and ADH (Figure 1.21.A.b) was detected.

Both herbicides provoked similar effects on the carbon metabolism and on the nitrogen metabolism. As a consequence of AHAS or EPSPS inhibition an increase in the total soluble sugars content (Figure 1.21.B.c and f), starch content (Figure 1.21.B.d and g) and total free amino acids content (Figure 1.21.B.e and h) was detected in the leaves and the roots *A. thaliana* wild-type plants.

These results validate the use of *A. thaliana* in the study of the physiological effects triggered by ABIH application in plants, since this species respond similar to other plant species.

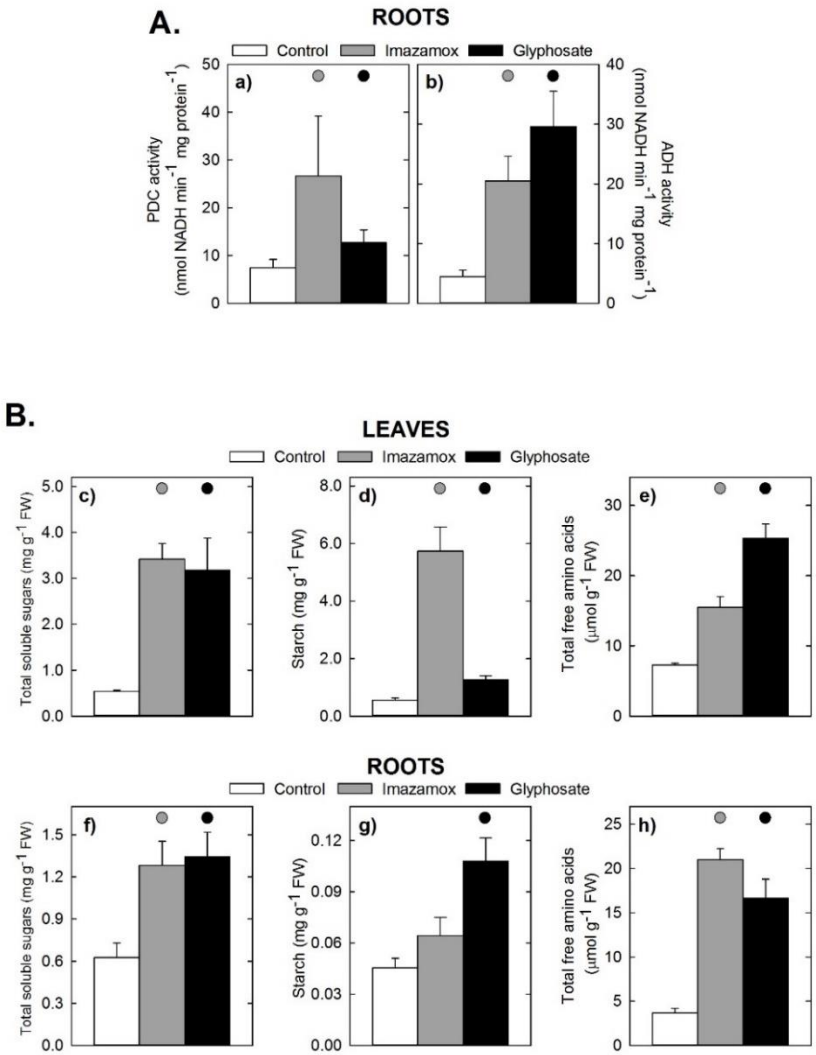


Figure 1.21. Imazamox and glyphosate effects on *Arabidopsis thaliana* Col-0 wild-type plants. **A.** *In vitro* enzymatic activities of Pyruvate decarboxylase (PDC) (a) and alcohol dehydrogenase (ADH) (b) in the roots. **B**. Total soluble sugars (c and f), starch (d and g) and total free amino acids (e and h) contents in the leaves and the roots. Values represent the mean ± SE (n = 5 biological replicates). Significant variations are marked with ● for differences between control and imazamox treated plants, and with ● for differences between control and glyphosate treated plants (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).

c) Herbicide effects on wild-type *A. thaliana* and *adh1* mutants

To evaluate the importance of the fermentation pathway in the response of the plants to ABIH application, the effects of IMX and GLP on *A. thaliana* mutants lacking the *ADH1* gene were compared with the effects provoked in wild-type plants. In particular, T-DNA knockout mutants for the *ADH1* gene were used (Banti et al., 2008). Previous studies demonstrated that *adh* null mutants have less tolerance to hypoxia than wild-type plants (Dolferus et al., 1997; Ellis et al., 1999; Ismond et al., 2003).

The *in vitro* enzymatic activities of PDC and ADH and the soluble sugars, starch, individual free amino acids, quinate and shikimate contents were monitored in the leaves and the roots of hydroponically grown plants of each studied genotypes (untreated or treated for three days with IMX or GLP). Moreover, to evaluate whether IMX and GLP share more common physiological effects than the previously described ones, the content of different organic acids from the TCA cycle and the glutathione contents were also measured. The effect of herbicides on growth were also monitored in *A. thaliana* seedlings of both genotypes grown on agar-containing vertical plates.

c. 1) Characterization of adh1 mutants under control conditions

First, untreated control plants of both genotypes used in this work were compared in order to ascertain whether or not any differences were due to the treatment or to the lack of the ethanol fermentation pathway.

No phenotypical differences were found between the different lines used and for almost all the parameters measured no differences between the untreated plants of each genotypes were found, except for the parameters presented in the Table 1.2. The differences between the untreated plants of the two studied genotypes are expressed in the corresponding figures with the symbol ∇.

	LEAVES	ROOTS
Free amino acids	Amides % respect to the total Serine Lysine	Acids % respect to the total
Carbohydrates		Fructose Glucose Total soluble sugars
Organic acids		Malate Pyruvate

Table 1.2. Parameters where differences were found between the untreated wild-type *Arabidopsis thaliana* and *adh1* mutants.

c. 2) Ethanol fermentation

The *in vitro* activities of PDC and ADH were measured in the leaves and the roots of IMX- or GLP-treated (for 3 days) wild-type *A. thaliana* and *adh1* mutant plants (Figure 1.22).

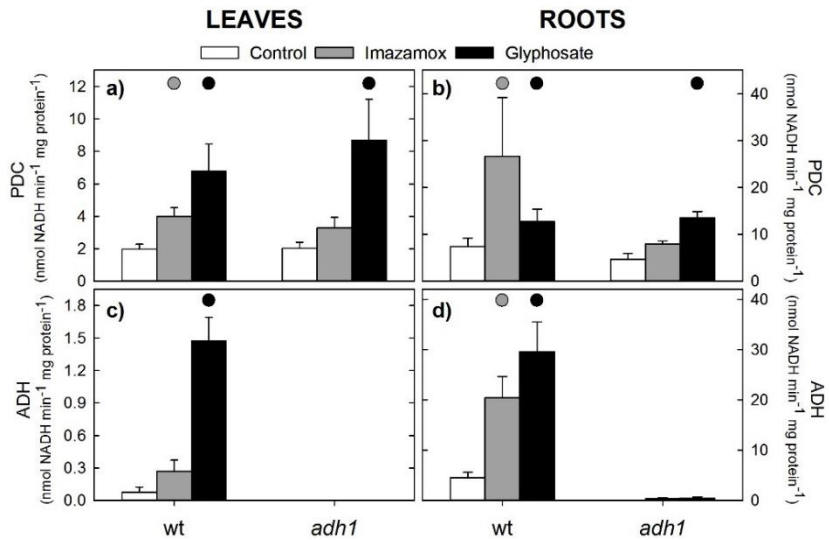


Figure 1.22. The *in vitro* activities of pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) in the leaves (a and c) and the roots (b and d) of wild-type *Arabidopsis thaliana* Col-0 and adh1 mutant plants, untreated (control) or treated with imazamox or glyphosate for 3 days. Values represent the mean ± SE (n = 5 biological replicates). Significant variations are marked with ● for differences between control and imazamox-treated plants, and with ● for differences between control and glyphosate-treated plants (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).

The effect provoked by both herbicides was more severe in the roots comparing to the effects provoked on the leaves. In the leaves of wild-type plants, while IMX only increased PDC activity, GLP increased the activity of both PDC and ADH in this organ. However, both IMX and GLP provoked an induction of PDC and ADH activities in the roots of wild-type plants.

Regarding the *adh1* mutants, as expected, no ADH activity was detected in the leaves and the roots of these plants. The PDC activity of *adh1* mutants increased as a consequence of GLP application in both organs, but not after IMX treatment.

c. 3) Shoot and root growth

In order to monitor herbicide effects on the growth, wild-type *A. thaliana* and *adh1* mutant seedlings were grown on agar containing vertical plates. Preliminary studies were conducted to determine the corresponding herbicides doses that was not too aggressive and killed the plant immediately, but that was sufficiently aggressive to have an effect on the plant growth. Finally, 0.005 mg active ingredient L^{-1} of IMX (0.016 µM) and 0.25 mg active ingredient L^{-1} of GLP (1.1 µM) were selected as herbicide doses. Both herbicide doses provoked similar effects on the plant growth and thus, were comparable among each other.

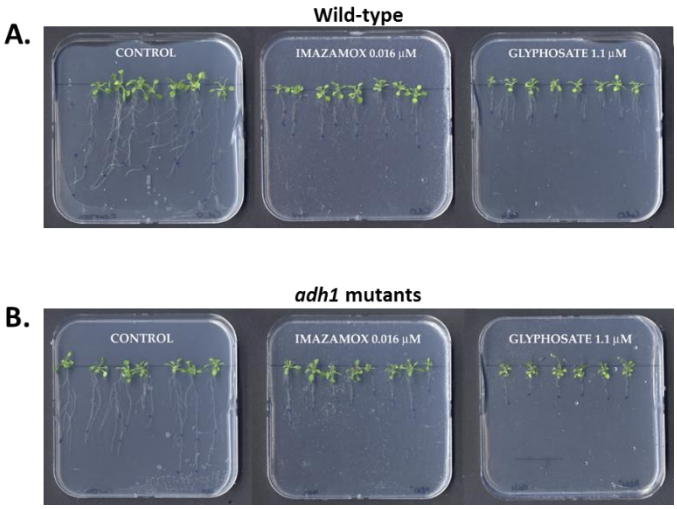


Figure 1.23. *Arabidopsis thaliana* wild-type (A) and *adh1* mutant (B) plants untreated, treated with 0.016 µM of imazamox or treated with 1.1 µM of glyphosate.

A growth arrest was observed in both the shoots and the roots of IMX or GLP treated wild-type and *adh1* mutants plants (Figure 1.23). The effects provoked on both genotypes by both herbicides were similar, thus, the lack of the *ADH1* gene did not modify the effect of the herbicides on the plant growth.

c. 4) Free amino acid content

Levels of the free amino acids (including the non-proteinogenic amino acid γ-aminobutyric acid (GABA)) were monitored in the leaves and the roots of control and herbicide-treated *A. thaliana* plants.

An increase in the total free amino acid content was observed in both the leaves and the roots of IMX or GLP treated plants (Figure 1.24). While GLP affected the leaves more than IMX did, both herbicides had a similar effect in the roots of treated plants. The accumulation of total free amino acids observed in the roots of *adh1* mutant plants, was slightly alleviated comparing to the accumulation observed in the roots of wild-type plants.

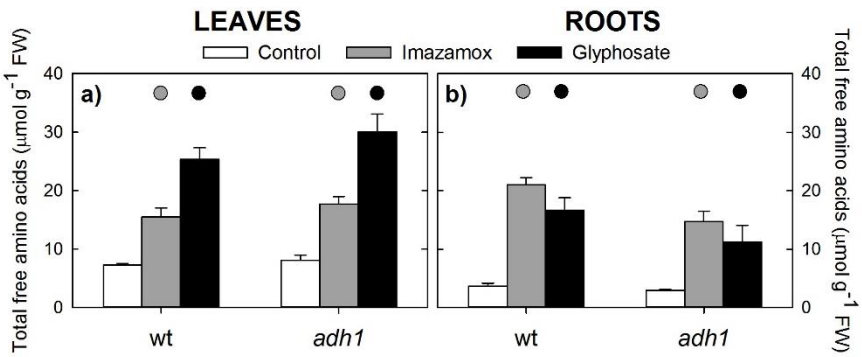


Figure 1.24. Total free amino acid content in the leaves (a) and the roots (b) of wild-type (wt) *Arabidopsis thaliana* Col-0 and *adh1* mutant plants, untreated (control) or treated with imazamox or glyphosate for 3 days. Values represent the mean ± SE (n = 5 biological replicates). Significant variations are marked with ● for differences between control and imazamox-treated plants, and with ● for differences between control and glyphosate-treated plants (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).

The content of each individual amino acid was also measured and, as the impact of changes of specific amino acids could be masked by the general

increase of the total free amino acid pool content, each specific group of amino acids has also been expressed as a the percentage of the total free amino acids. The amino acids are presented and grouped by the main biosynthetic pathways, and they are presented in different colours. A simplified overview of all the amino acid biosynthesis is shown in Figure 1.25.

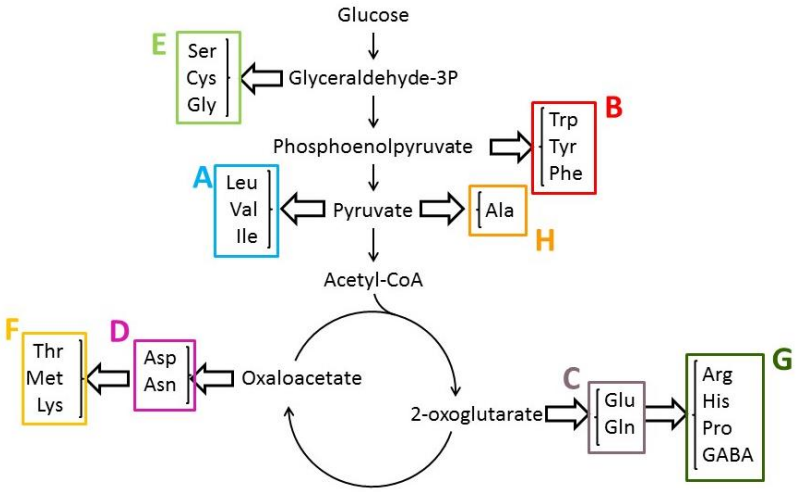


Figure 1.25. Overview of amino acid biosynthesis in plants (modified from Coruzzi and Last, 2000).

Regarding the BCAAs (Figure 1.26), a different pattern in the BCAA content was observed following IMX or GLP treatment and both leaves and roots respond in a similar way to the herbicides. Taking into account that IMX inhibits the biosynthesis of BCAAs, a deep decrease on BCAA content was expected. However, looking at the individual amino acid content, only the amount of Val was reduced in the leaves and the roots of IMX treated plants. The percentage of BCAA with respect to the total amino acid content also decreased in both organs after IMX treatment. On the other hand, a substantially high increase in BCAA contents (individual BCAA content, total BCAA content and individual BCAA content with respect to the total free amino acids) was observed in both organs following GLP treatment. When comparing both

studied genotypes no remarkable differences were detected regarding the BCAA contents.

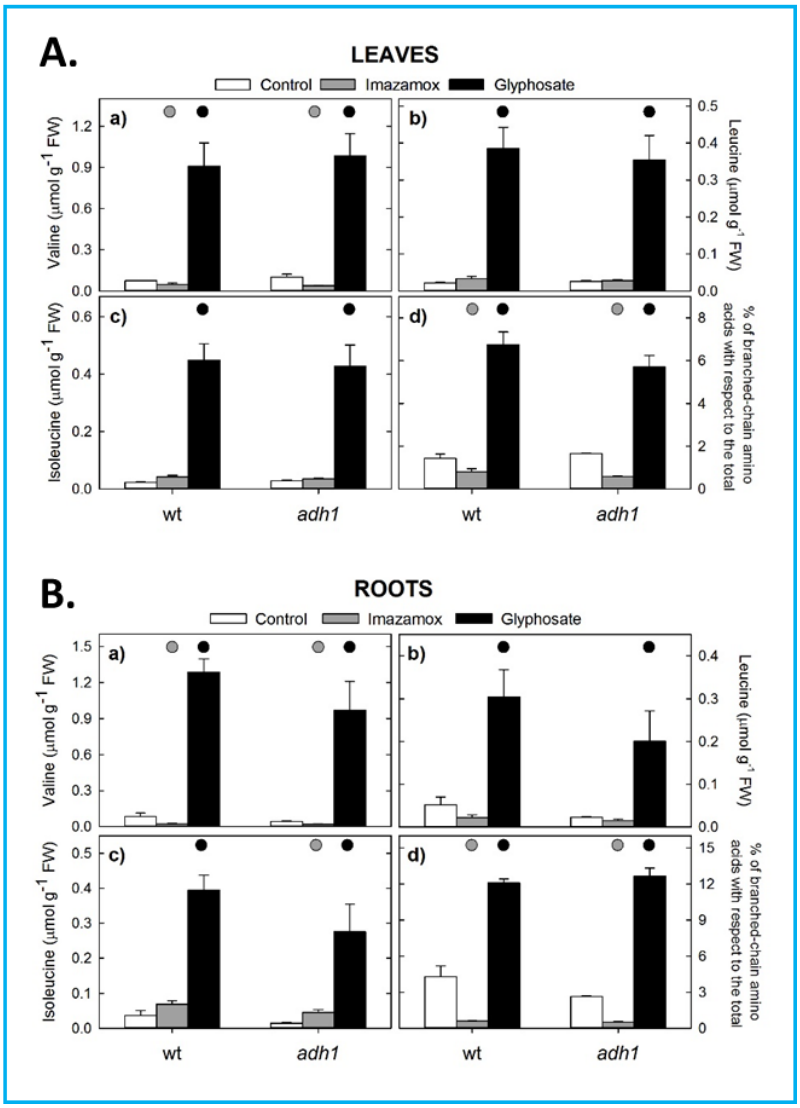


Figure 1.26. Branched-chain amino acid content in the leaves (A) and the roots (B) of wild-type (wt) *Arabidopsis thaliana* Col-0 and *adh1* mutant plants, untreated (control) or treated with imazamox or glyphosate for 3 days. Values represent the mean ± SE (n = 5 biological replicates). Significant variations are marked with ● for differences between control and imazamox-treated plants, and with ● for differences between control and glyphosate-treated plants (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).

In general, an increase in the aromatic amino acid levels was observed in the leaves and the roots after herbicide application (Figure 1.27). Although an accumulation of the aromatic amino acids was detected in leaves and roots of IMX treated plants, a decrease in the aromatic amino acids represented as percentage of the total amino acids was observed in the roots. On the other hand, as GLP inhibits the EPSPS activity, a decrease in the aromatic amino acid content is expected after GLP application. However, in general, aromatic amino acid content increased in both the leaves and the roots of GLP treated plants, although no changes in the aromatic amino acids levels represented as percentage of the total amino acids were observed in the roots of GLP treated plants. The same pattern was observed for both genotypes, except that the accumulation of phenylalanine observed in the roots after IMX treatment was not detected in the *adh1* mutants.

A general increase in the levels of each individual acidic (glutamate and aspartate) and amide (glutamine and asparagine) amino acids was detected in leaves (Figure 1.28) and roots (Figure 1.29) of IMX or GLP treated plants. However, the percentage of acidic amino acids with respect to the total amino acids decreased in the leaves and the roots after both herbicide treatment. In contrast, the amide amino acid content with respect to the total free amino acid content increased in the leaves with GLP and in the roots with IMX. Both studied genotypes showed similar patterns, however, GLP effect was slightly alleviated in the roots of the *adh1* mutant, where no increase in glutamate, glutamine and aspartate were observed.

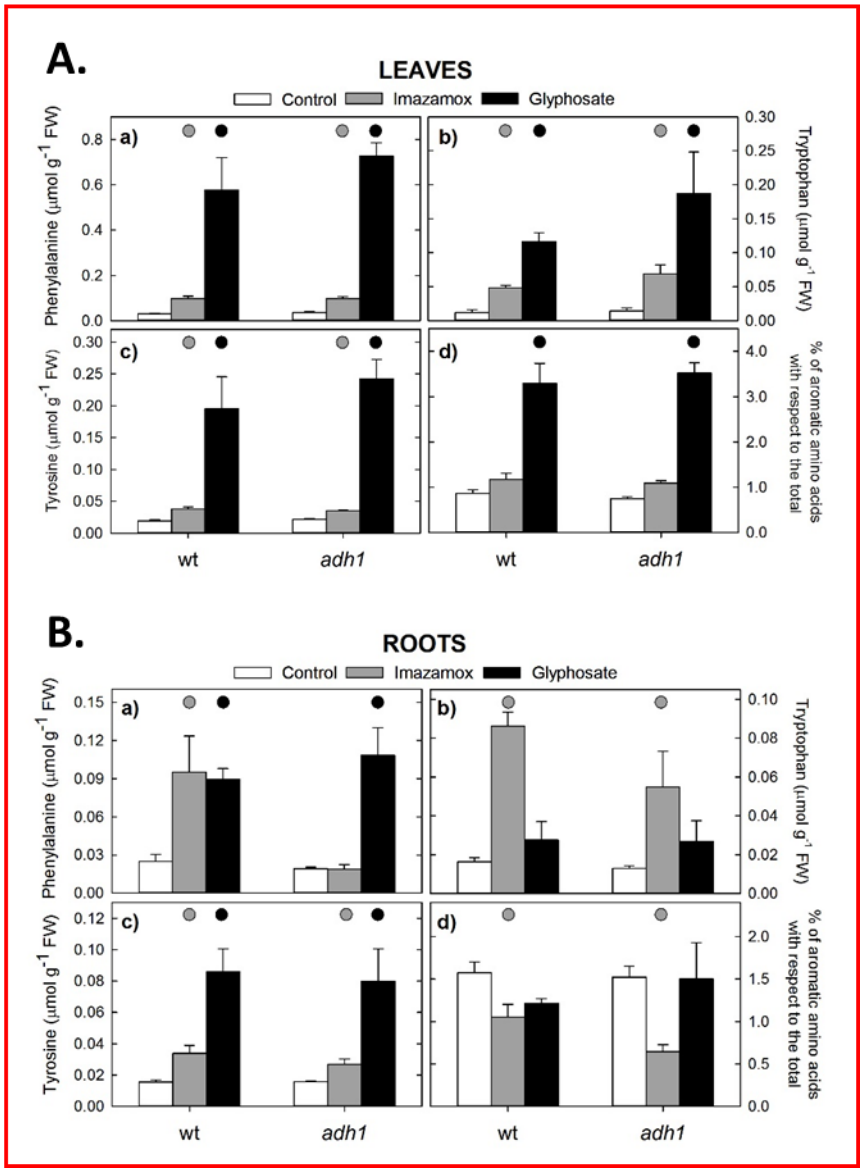


Figure 1.27. Aromatic amino acid content in the leaves (A) and the roots (B) of wild-type (wt) *Arabidopsis thaliana* Col-0 and *adh1* mutant plants, untreated (control) or treated with imazamox or glyphosate for 3 days. Values represent the mean ± SE (n = 5 biological replicates). Significant variations are marked with ● for differences between control and imazamox-treated plants, and with ● for differences between control and glyphosate-treated plants (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).

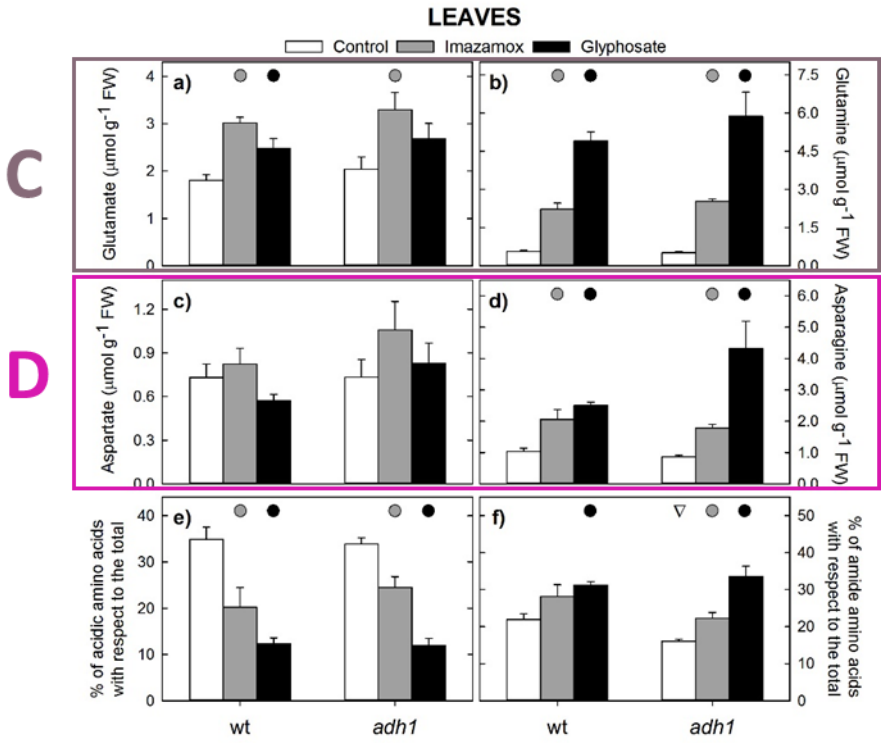


Figure 1.28. Acidic (glutamate and aspartate) and amide (glutamine and asparagine) amino acid content in leaves of wild-type (wt) *Arabidopsis thaliana* Col-0 and *adh1* mutant plants, untreated (control) or treated with imazamox or glyphosate for 3 days. Values represent the mean ± SE (n = 5 biological replicates). Significant differences between the untreated plants of both genotypes are marked with ∇ (*t*-Test, $p < 0.05$). Significant variations are marked with ● for differences between control and imazamox-treated plants, and with ● for differences between control and glyphosate-treated plants (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).

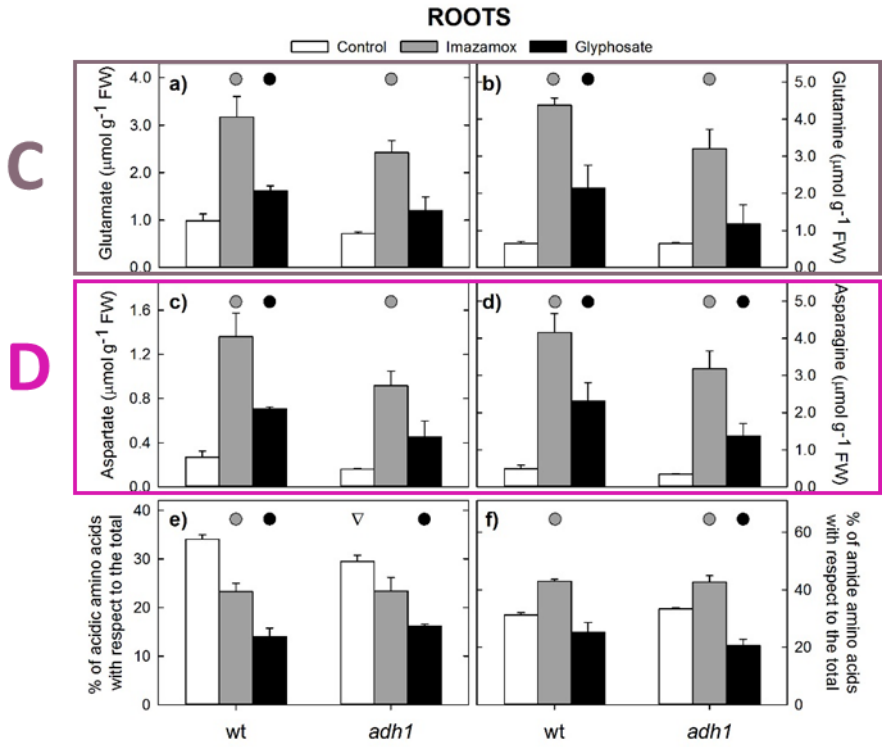


Figure 1.29. Acidic (glutamate and aspartate) and amide (glutamine and asparagine) amino acid content in the roots of wild-type (wt) *Arabidopsis thaliana* Col-0 and *adh1* mutant plants, untreated (control) or treated with imazamox or glyphosate for 3 days. Values represent the mean ± SE (n = 5 biological replicates). Significant differences between the untreated plants of both genotypes are marked with ▽ (*t*-Test, $p < 0.05$). Significant variations are marked with ● for differences between control and imazamox-treated plants, and with ● for differences between control and glyphosate-treated plants (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).

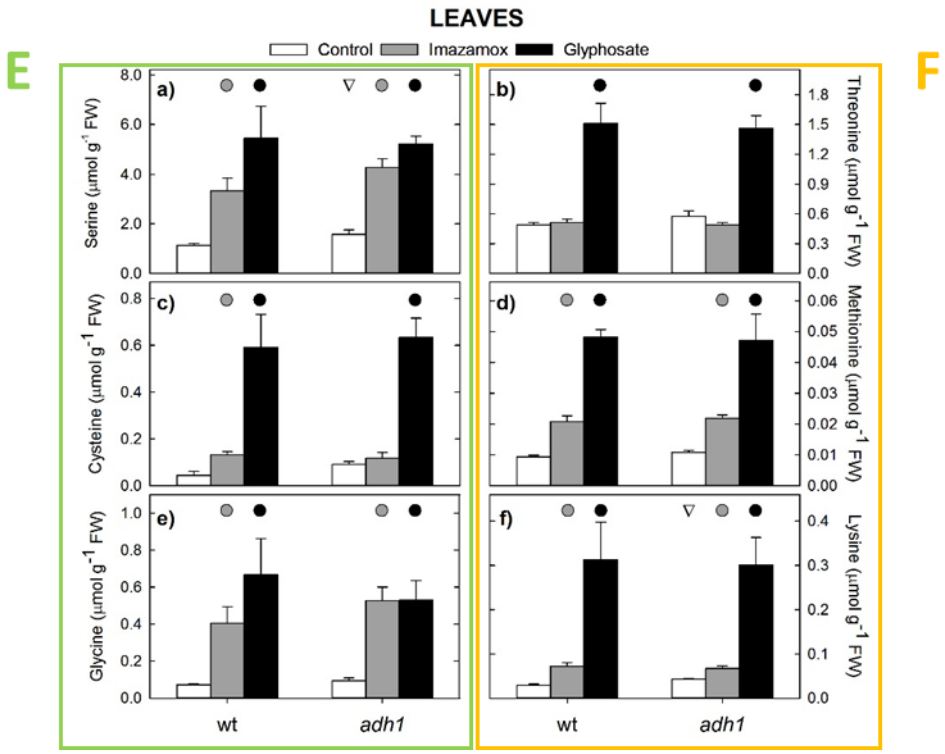


Figure 1.30. Serine (a), threonine (b), cysteine (c), methionine (d), glycine (e) and lysine (f) content in the leaves of wild-type (wt) *Arabidopsis thaliana* Col-0 and *adh1* mutant plants, untreated (control) or treated with imazamox or glyphosate for 3 days. Values represent the mean ± SE (n = 5 biological replicates). Significant differences between the untreated plants of both genotypes are marked with ▽ (*t*-Test, $p < 0.05$). Significant variations are marked with ● for differences between control and imazamox-treated plants, and with ● for differences between control and glyphosate-treated plants (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).

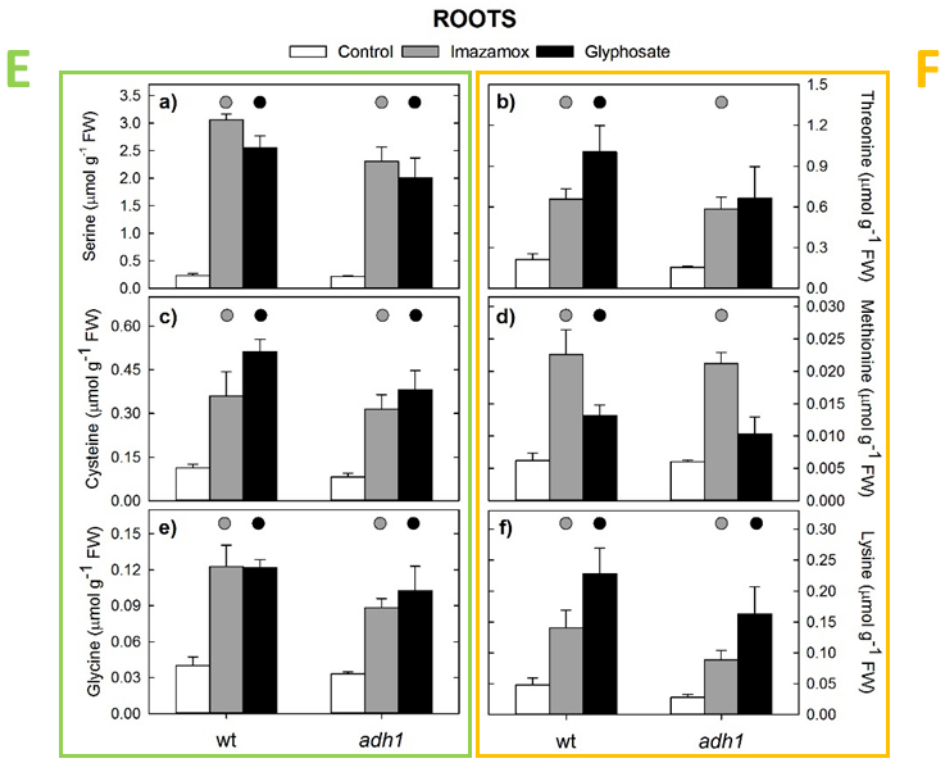


Figure 1.31. Serine (a), threonine (b), cysteine (c), methionine (d), glycine (e) and lysine (f) content in the roots of wild-type (wt) *Arabidopsis thaliana* Col-0 and *adh1* mutant plants, untreated (control) or treated with imazamox or glyphosate for 3 days. Values represent the mean ± SE (n = 5 biological replicates). Significant variations are marked with ● for differences between control and imazamox-treated plants, and with ● for differences between control and glyphosate-treated plants (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).

Both herbicides provoked an accumulation of serine, threonine, cysteine, methionine, glycine and lysine in leaves (Figure 1.30) and roots (Figure 1.31) of *A. thaliana* wild-type plants, with the exception of IMX on the leaf threonine content. Similar pattern was observed in *adh1* mutants, but the effect of GLP was alleviated in the roots of this plants, where no accumulation of threonine and methionine was observed.

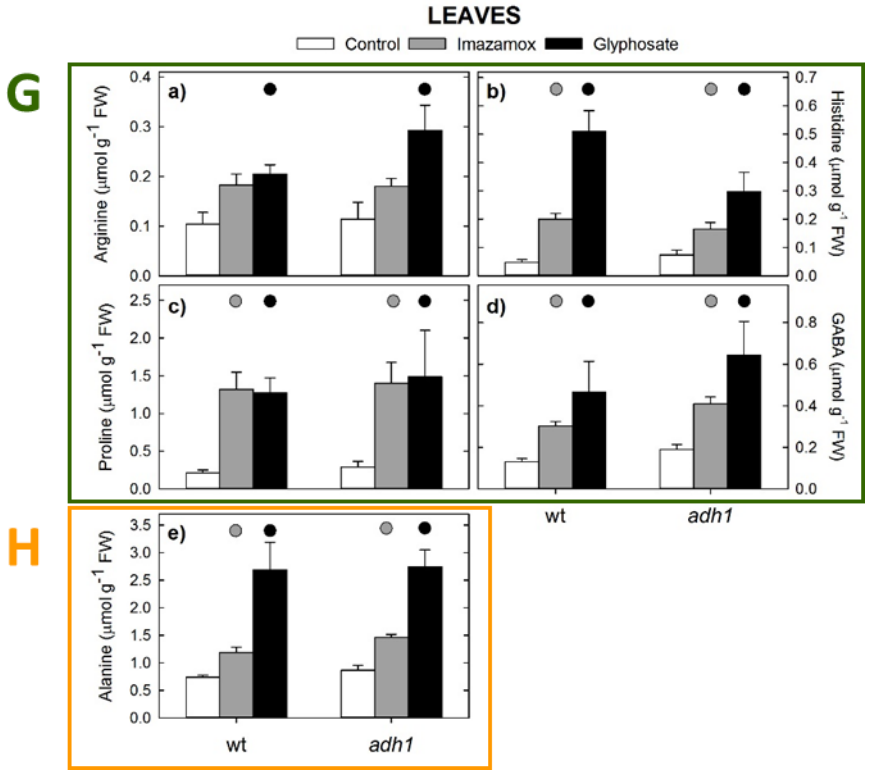


Figure 1.32. Arginine (a), histidine (b), proline (c), γ-aminobutyric acid (GABA) (d) and alanine (e) content in the leaves of wild-type (wt) *Arabidopsis thaliana* Col-0 and *adh1* mutant plants, untreated (control) or treated with imazamox or glyphosate for 3 days. Values represent the mean ± SE (n = 5 biological replicates). Significant variations are marked with ● for differences between control and imazamox-treated plants, and with ● for differences between control and glyphosate-treated plants (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).

An increase in the arginine, histidine, proline, γ-aminobutyric acid and alanine content was found in the leaves (Figure 1.32) and the roots (Figure 1.33) of IMX- or GLP-treated *A. thaliana* plants, with exception of IMX in the leaves arginine content. The same pattern was observed in the plants of both studied genotypes.

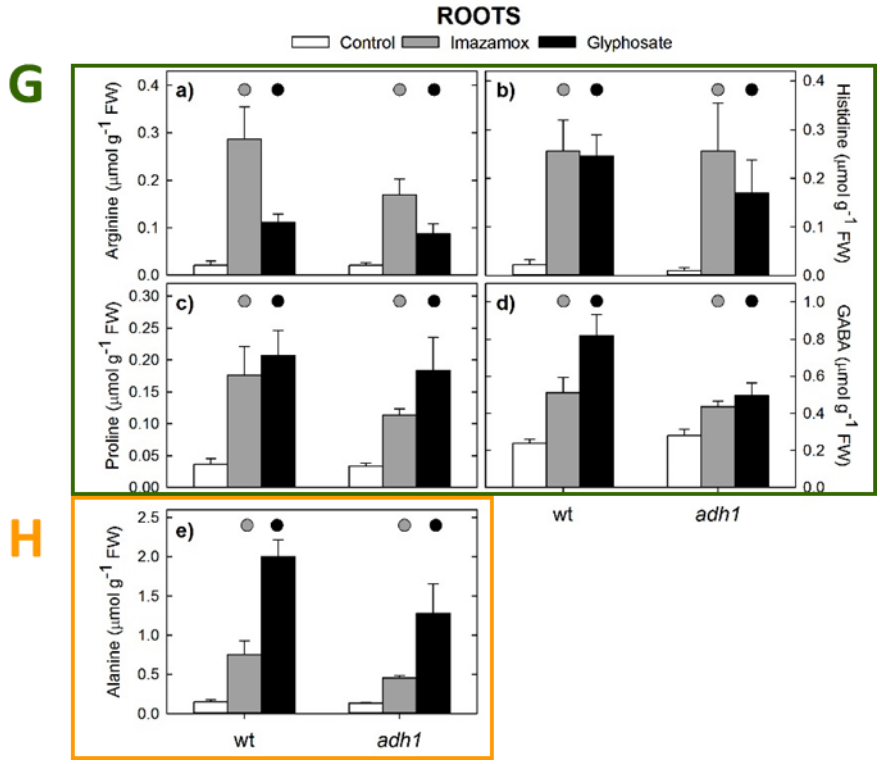


Figure 1.33. Arginine (a), histidine (b), proline (c), γ-aminobutyric acid (GABA) (d) and alanine (e) content in the roots of wild-type (wt) *Arabidopsis thaliana* Col-0 and *adh1* mutant plants, untreated (control) or treated with imazamox or glyphosate for 3 days. Values represent the mean ± SE (n = 5 biological replicates). Significant variations are marked with ● for differences between control and imazamox-treated plants, and with ● for differences between control and glyphosate-treated plants (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).

c. 5) Carbohydrate content

Effects of IMX and GLP on the carbohydrate (soluble sugars and starch) content were evaluated in the leaves (Figure 1.34) and the roots (Figure 1.35) of *A. thaliana* wild-type and *adh1* mutants. An accumulation of fructose, glucose and starch was found in the leaves of IMX or GLP treated wild-type plants, sucrose was also accumulated after GLP application. However, in *adh1* mutants the herbicide effects were alleviated, mainly in GLP treated plants, where only fructose was accumulated and no increase in the glucose, sucrose, total soluble sugars and starch contents was detected.

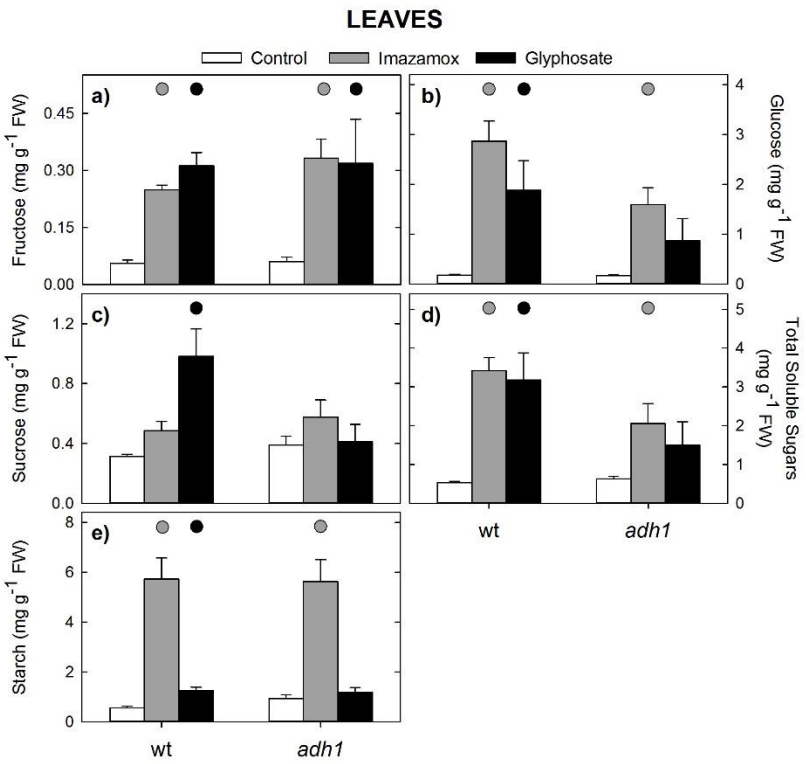


Figure 1.34. The carbohydrate content in the leaves of wild-type (wt) *Arabidopsis thaliana* Col-0 and adh1 mutant plants, untreated (control) or treated with imazamox or glyphosate for 3 days. Values represent the mean ± SE (n = 5 biological replicates). Significant variations are marked with ● for differences between control and imazamox-treated plants, and with ● for differences between control and glyphosate-treated plants (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).

An increase in the levels of total soluble sugars was detected in the roots of IMX or GLP treated wild-type *A. thaliana* plants. This increase was due to an accumulation of glucose in the case of IMX treated plants, and due to an accumulation of sucrose for GLP treated plants. Starch was also accumulated in the roots of GLP treated plants. The effects of the herbicides were alleviated in the *adh1* mutants, and no increase in total soluble sugars and starch content were detected after IMX or GLP treatment.

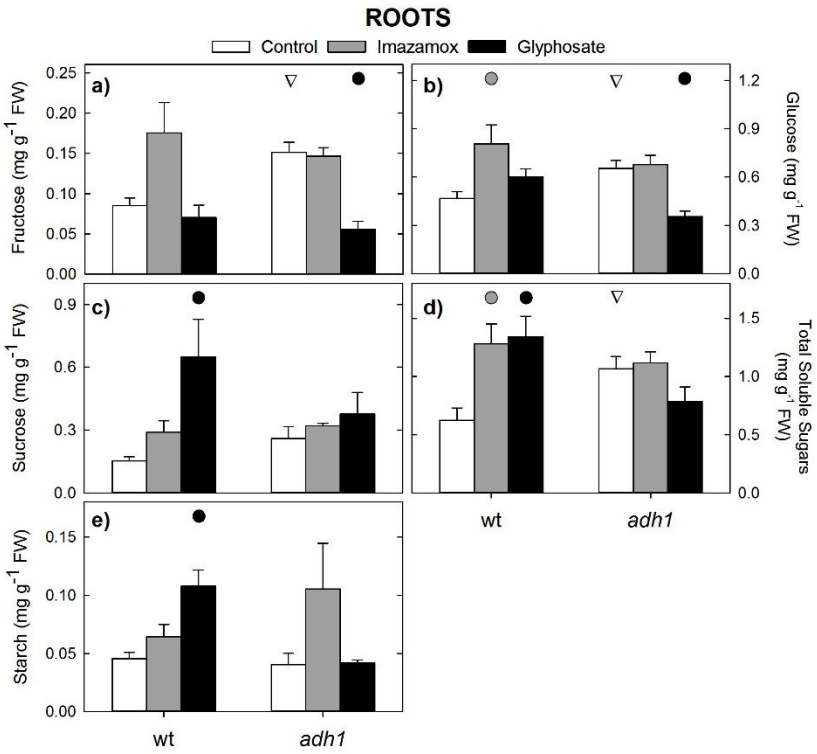


Figure 1.35. The carbohydrate content in the roots of wild-type (wt) *Arabidopsis thaliana* Col-0 and *adh1* mutant plants, untreated (control) or treated with imazamox or glyphosate for 3 days. Values represent the mean ± SE (n = 5 biological replicates). Significant differences between the untreated plants of both genotypes are marked with ∇ (*t*-Test, $p < 0.05$). Significant variations are marked with ● for differences between control and imazamox-treated plants, and with ● for differences between control and glyphosate-treated plants (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).

c. 6) Organic acids content

An increase in the pyruvate and lactate content was observed in the leaves of wild-type plants after GLP treatment (Figure 1.36.a and b). By contrast, in the leaves of *adh1* mutants no changes in the pyruvate and lactate content were observed. Regarding the organic acids that participate in the TCA cycle (Figure 1.36 c-f), while a decrease in the citrate content was observed in the leaves of wild-type plants after both herbicide treatment, an accumulation of α-ketoglutarate, succinate and malate was observed. In the plants lacking the

ADH1 gene, the herbicide effect was slightly alleviated and no changes in the α-ketoglutarate content were observed after herbicide treatment.

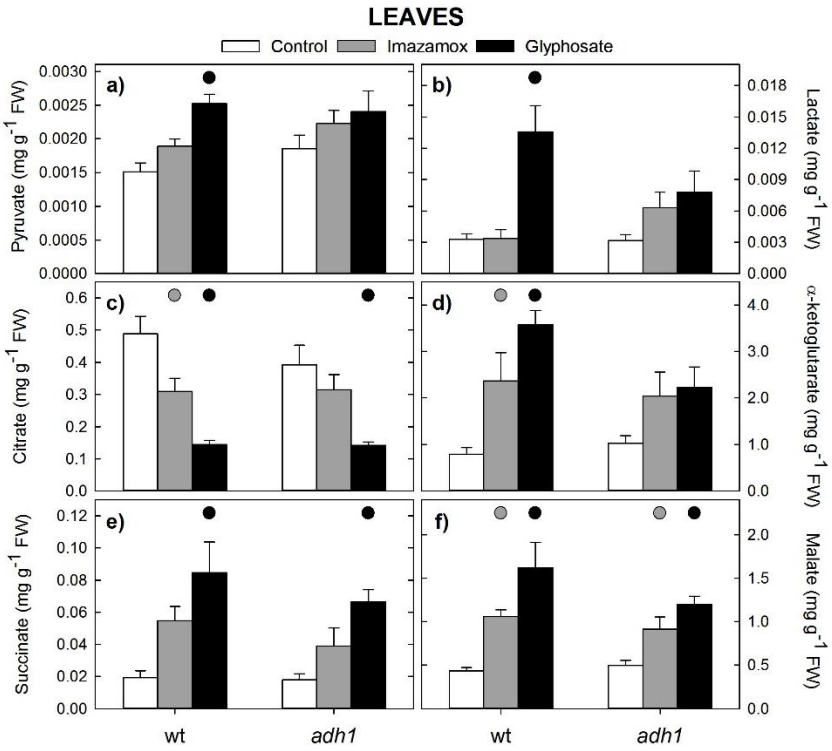


Figure 1.36. Organic acid content in the leaves of wild-type (wt) *Arabidopsis thaliana* Col-0 and *adh1* mutant plants, untreated (control) or treated with imazamox or glyphosate for 3 days. Values represent the mean ± SE (n = 5 biological replicates). Significant variations are marked with ● for differences between control and imazamox-treated plants, and with ● for differences between control and glyphosate-treated plants (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).

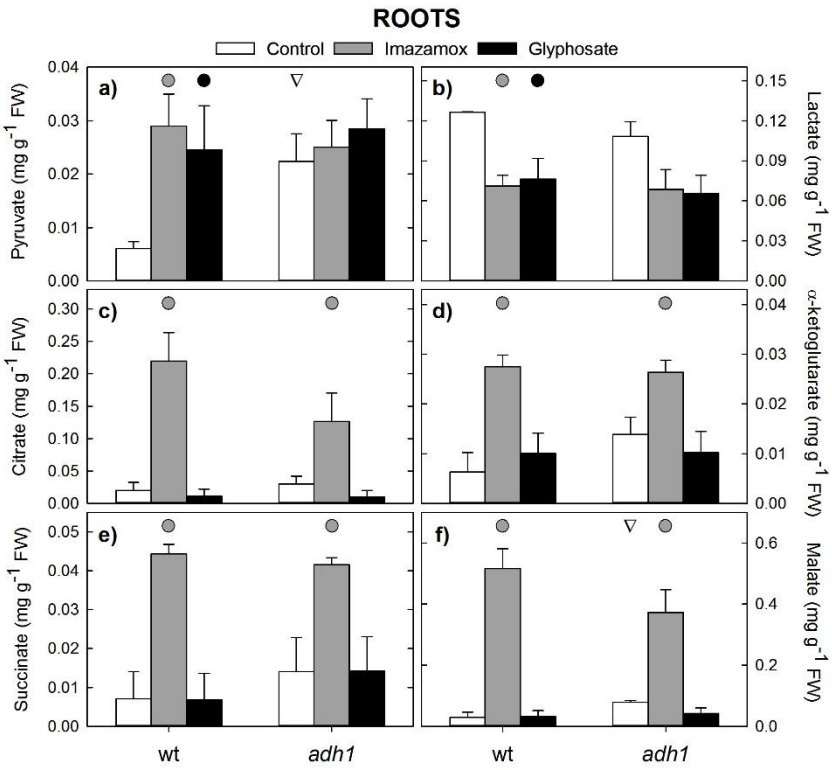


Figure 1.37. Organic acid content in the roots of wild-type (wt) *Arabidopsis thaliana* Col-0 and *adh1* mutant plants, untreated (control) or treated with imazamox or glyphosate for 3 days. Values represent the mean ± SE (n = 5 biological replicates). Significant differences between the untreated plants of both genotypes are marked with ∇ (*t*-Test, $p < 0.05$). Significant variations are marked with ● for differences between control and imazamox-treated plants, and with ● for differences between control and glyphosate-treated plants (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).

An accumulation of pyruvate (Figure 1.37.a) and a decrease in the lactate (Figure 1.37.b) content were observed after both herbicide treatments in the roots of wild-type plants. However, herbicides did not change the levels of these metabolites in the *adh1* mutants. IMX provoked an accumulation of citrate, α-ketoglutarate, succinate and malate in the roots of wild-type (Figure 1.37 c, d, e, and f). In contrast, GLP had no effect in the content of these metabolites. The same pattern observed in wild-type plants was detected in the levels of the organic acids involved in the TCA cycle in the *adh1* mutant plants

The shikimate and quinate contents were measured in IMX- or GLP-treated (for three days) plants (Figure 1.38). Regarding the shikimate content (Figure 1.38.a and b), as a consequence of EPSPS inhibition an accumulation of shikimate (the EPSPS substrate) was observed in the leaves and roots of wild-type plants. The same pattern was observed in the *adh1* mutants.

As for the quinate content (Figure 1.38.c and d), an accumulation of quinate was observed in the leaves of wild-type plants as a consequence of EPSPS inhibition, by contrast, in the roots, quinate was accumulated after AHAS inhibition. The plants lacking the *ADH1* gene presented the same pattern observed in the wild-type plants.

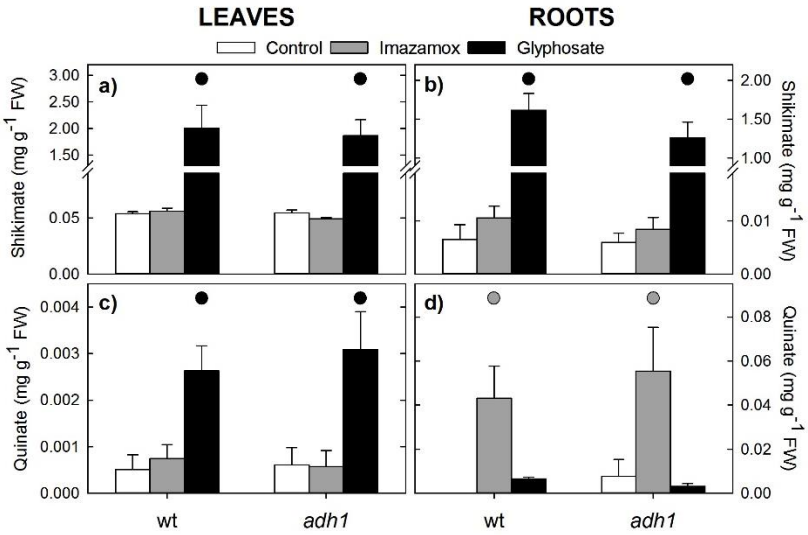


Figure 1.38. Shikimate (a and b) and quinate (c and d) content in the leaves and the roots of wild-type (wt) *Arabidopsis thaliana* Col-0 and *adh1* mutant plants, untreated (control) or treated with imazamox or glyphosate for 3 days. Values represent the mean ± SE (n = 5 biological replicates). Significant variations are marked with ○ for differences between control and imazamox-treated plants, and with ● for differences between control and glyphosate-treated plants (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).

c. 7) Glutathione content

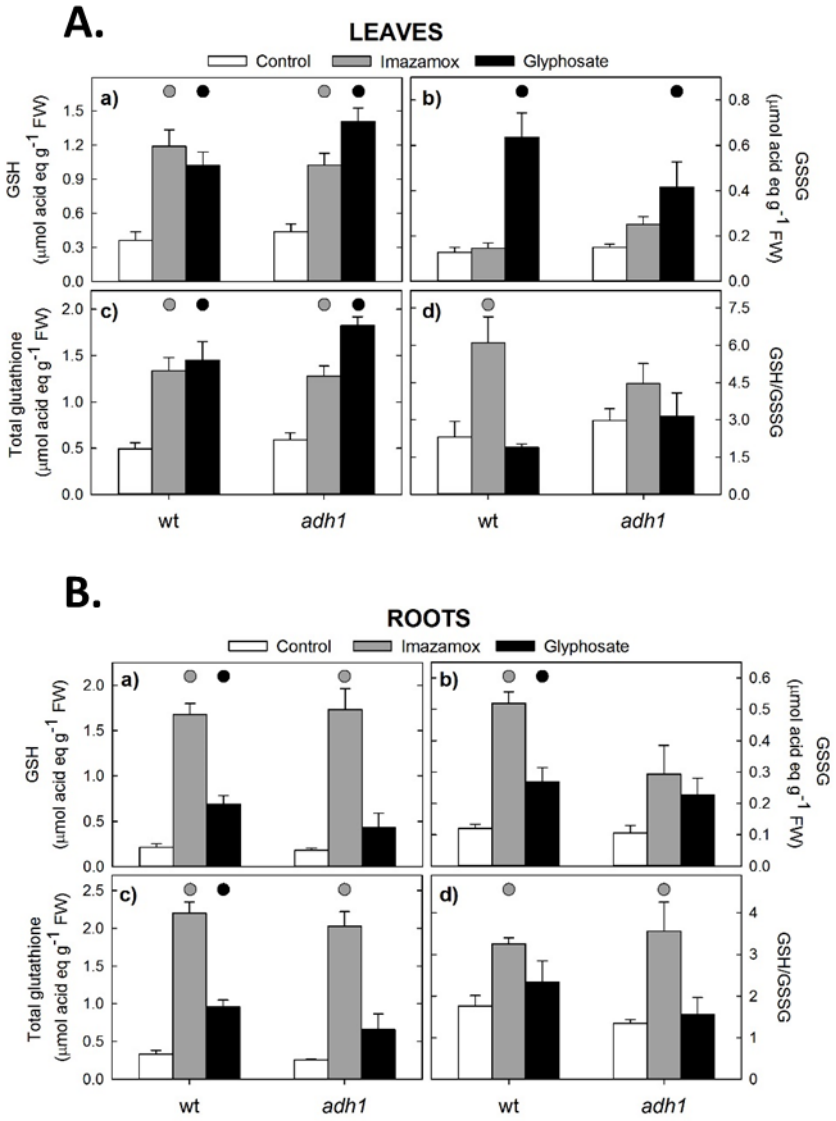


Figure 1.39. Reduced glutathione content (GSH) (a), oxidized glutathione content (GSSG) (b), sum of GSH and GSSG (c) and ratio GSH/GSSG (d) in the leaves (A) and the roots (B) wild-type (wt) *Arabidopsis thaliana* Col-0 and *adh1* mutant plants, untreated (control) or treated with imazamox or glyphosate for 3 days. Values represent the mean ± SE (n = 5 biological replicates). Significant variations are marked with ● for differences between control and imazamox-treated plants, and with ● for differences between control and glyphosate-treated plants (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).

The contents of the reduced glutathione (GSH) and oxidized glutathione (GSSG) were measured in the leaves (Figure 1.39.A) and the roots (Figure 1.39.B) of wild-type *A. thaliana* and *adh1* mutant plants treated for 3 days with IMX or GLP. The total glutathione (sum of reduced and oxidized glutathione) content and the ratio of reduced/oxidized glutathione are also represented.

An increase in the GSH content was observed in the leaves and the roots of IMX- or GLP-treated plants. The GSSG in the leaves only increased after GLP treatment, but in the roots it was accumulated after both herbicide application. Regarding the total glutathione content, it increased in the leaves and the roots after both herbicides application. As for the GSH/GSSG ratio, while an increase of this parameter was observed after IMX treatment in both studied organs, GSH/GSSG ratio was not affected by GLP.

In *adh1* mutants few differences were observed with the results obtained in wild-type plants. In the leaves, IMX did not provoke an increase in the GSH/GSSG ratio. Moreover, contrary to what it was observed in the wild-type plants, in the roots of mutant plants no accumulation of GSH and total glutathione after GLP application was detected; and the GSSG content did not increase after IMX or GLP treatment.

1. 4. II. 2. DISCUSSION (PART II)

An induction of ethanol fermentation has been described in plants as a consequence of AHAS or EPSPS inhibition, however, its role has not been in depth studied yet.

To evaluate the importance of the fermentation pathway in the response of the plants to ABIH application, the previously reported physiological effects triggered by this type of herbicides were monitored on *A. thaliana* mutants lacking the *ADH1* gene and they were compared with the effects provoked in wild-type plants. This way, it could be analysed whether the loss of the ethanol fermentation pathway modifies the response to ABIHs in the plants. In particular: the *in vitro* enzymatic activities of PDC and ADH, the shoot and root growth and the soluble sugars, starch, individual free amino acids, quinate and shikimate contents were monitored in the leaves and the roots of *A. thaliana* plants.

Additionally, to evaluate whether IMX and GLP provoke more common physiological effects than the previously described ones, the content of different organic acids of the TCA cycle and the glutathione contents (reduced and oxidized forms) were also measured and compared in both genotypes.

a) IMX and GLP cause similar effects on *A. thaliana* as on pea

An increase in the *in vitro* activity and the protein content of the PDC and ADH has been reported in pea roots treated with AHAS or EPSPS inhibitors (Gaston et al., 2002; Zabalza et al., 2007; Orcaray et al., 2012). Moreover, these effects were also detected in glufosinate-treated plants (Orcaray et al., 2012), a herbicide that inhibits glutamine biosynthesis (Duke, 1990). The induction of fermentation is a common effect detected after the inhibition of the biosynthesis of different amino acids. Our results confirm that ethanol fermentation is induced also in *A. thaliana* as a consequence of IMX or GLP application since an increase in the *in vitro* activities of the enzymes involved in the ethanol fermentation increased after AHAS or EPSPS inhibition (Figure 1.22).

The induction of ethanol fermentation has been deeply studied in the response of the plants to low-oxygen conditions and it has been related to a decrease in the energy charge provoked by the inhibition of respiration. Besides, this pathway has also been described to induce under different environmental stress conditions (e.g. drought, SO_2, low temperatures) (Kimmerer and Kozlowski, 1982; Tadege et al., 1999), however, the role of ethanol fermentation when the oxygen is not limiting has not been explained yet. Contrary to the response of the plants to low-oxygen conditions, in ABIH-treated plants the induction of fermentation was not related to a change in respiratory rates or to a decrease in the energy charge, as the energy charge actually increased (Zabalza et al., 2011; Orcaray et al., 2012). It has been proposed that fermentation has a general function in aerobic metabolism under stress conditions and that it might be an important switch in regulating carbohydrate metabolism (Tadege et al., 1999).

Growth arrest is another well-known physiological effect caused by ABIH and both shoot and root growth inhibition has been previously described in several plant species (e.g. soybean, rice, pea, common bean) following AHAS or EPSPS inhibition (Gaston et al., 2003; Zabalza et al., 2007; Qian et al., 2009; Orcaray et al., 2010; Qian et al., 2011a; García-Garijo et al., 2012; García-Garijo et al., 2013). Our results correlate with what it was observed for other species in previous studies, since both shoot and root growth inhibition was detected in *A. thaliana* treated with IMX or GLP (Figure 1.23).

An increase in the total free amino acid content has been described in different plant species treated with ABIH (Zabalza et al., 2006; Orcaray et al., 2010; García-Garijo et al., 2012; Zulet et al., 2013a). In this study, the total free amino acid content also increased in the leaves and the roots of IMX or GLP-treated wild-type *A. thaliana* plants (Figure 1.24). The increase in the total free amino acid content was a consequence of a general increase in the content of each individual amino acid. The increase in the total free amino acid content observed in ABIH-treated plants has been related to an increase in the soluble

protein degradation, indeed, soluble protein content decreases as a consequence of ABIH application (Zulet et al., 2013a). It has been proposed that after AHAS or EPSPS inhibition proteolysis is enhanced to degrade pre-existing proteins and prevent BCAA or AAA starvation (Zabalza et al., 2006). The proteolytic activities of ABIH-treated pea plants were investigated and although the expected increase in the proteolytic activities was not detected, several common effects were found after AHAS or EPSPS inhibition. Whereas the 26S proteasome system and the papain-like cysteine proteases were activated, the activities of the vacuolar processing enzymes, cysteine proteases and metacaspase 9 were decreased as a consequence of AHAS or EPSPS inhibition (Zulet et al., 2013a).

Regarding the individual amino acid content, similar results comparing to what it was seen for other species were obtained in this study. The content of the BCAA with respect to the total amino acid content increased as a consequence of EPSPS inhibition (Figure 1.26) as it occurred in the leaves of GLP-treated pea plants (Orcaray et al., 2010). Moreover, the content of AAA with respect to the total amino acids did not change after three days of EPSPS inhibition in the roots of *A. thaliana* (Figure 1.27.B.d). Similar results were also obtained for both plant species regarding the acidic and amide amino acids with respect to the total free amino acid content. In pea plants, a decrease in the acidic amino acids with respect to the total amino acids was detected in the leaves of imazethapyr- or GLP-treated plants three days after treatment (Orcaray et al., 2010); the same results were observed in the leaves of IMX- or GLP-treated *A. thaliana* plants (Figure 1.29). At day 3 of treatment, an increase in the amide amino acids with respect to the total free amino acid content was detected in the leaves of pea plants as a consequence of AHAS or EPSPS inhibition (Orcaray et al., 2010). In the leaves of *A. thaliana*, the amide amino acids with respect to the total free amino acids only increased after EPSPS inhibition (Figure 1.29).

Carbohydrates have also been described to accumulate in the leaves and the roots as a consequence of ABIH application (Gaston et al., 2002; Zabalza et al.,

2004; Qian et al., 2011b; Orcaray et al., 2012). In *A. thaliana* plants the carbohydrate content in the leaves and the roots increased after either IMX or GLP application (Figure 1.34 and 1.35). Carbohydrate accumulation in the sink and source organs has been explained as follows: since growth is arrested, the carbohydrates present in the sinks and sources are not metabolized and then are accumulated. The accumulation of carbohydrates has been observed to occur first in the sinks and later in the sources (Zabalza et al., 2004). As a consequence of carbohydrates accumulation in the sinks, the sugar-gradient necessary for long-distance transport is abolished and thus, the phloem transport is inhibited leading to carbohydrate accumulation in the sources (Zabalza et al., 2004; Orcaray et al., 2012).

Shikimate is a metabolic intermediate in the aromatic amino acid biosynthesis (see Figure C.5 for biosynthetic pathway) known to accumulate as a consequence of EPSPS inhibition (Lydon and Duke, 1988; Hernandez et al., 1999; Orcaray et al., 2010) and it has been proposed that it could be used to identify GLP-resistant plants (Shaner et al., 2005). In our study shikimate accumulated after GLP treatment in both leaves and roots of *A. thaliana* plants (Figure 1.38), these results correlate with the results obtained for other plant species. The blockage of shikimate pathway at the EPSPS level deregulates the modulation of carbon flow into the pathway (Jensen, 1986), causing a massive carbon entrance that accumulates in compounds upstream of the EPSP inhibition point (i.e. shikimate, protocatechuic and gallic acids). It has been proposed that accumulation of shikimate and protocatechuic acid owing to EPSPS inhibition by GLP would divert most PEP from the glycolytic flow to the shikimate pathway (De María et al., 2006), because both acids are potent PEP carboxylase inhibitors.

Quinate, another intermediate of the shikimate pathway (see Figure C.5 for biosynthetic pathway), has also been described to accumulate as a consequence of AHAS or EPSPS inhibition (Orcaray et al., 2010). In our study, quinate accumulation was observed in the leaves of GLP-treated plants, while in the

roots it was accumulated after IMX application (Figure 1.38). It has been observed that quinate can mimic the herbicidal effect and it has been proposed as a natural compound in weed management (Orcaray et al., 2010; Zulet et al., 2013b). Exogenously supplied quinate (to the leaves or to the roots) deregulated the shikimate pathway in the plants and mimicked some physiological effects described for the ABIH (Zulet et al., 2013b) and moreover, the application of quinate through the nutrient solution provoked plant death (Zulet et al., 2013b).

Collectively these results indicate that *A. thaliana* shows the typical physiological markers as toxic consequences of ABIH, which have been reported previously in other species with some deviations. The confirmed effects on *A. thaliana* wild-type plants support the use of mutants to evaluate the physiological effects of these herbicides in mutant genotypes that lack part of the ethanol fermentation pathway. These physiological effects are used in the present work as markers of herbicide effect, and therefore toxicity has been compared through these markers.

b) Both IMX and GLP provoke more common physiological effects than the previously described ones

Although they inhibit different metabolic pathways, several common physiological effects have been described in plants as a consequence ABIH application, and they have also been observed in this study. Additionally, we detected that both IMX and GLP provoked more common effects in *A. thaliana* than the previously described ones.

An accumulation of pyruvate has been detected in the leaves and the roots of IMX- or GLP-treated *A. thaliana* plants (Figure 1.36 and 1.37) as has been reported before in pea and soybean roots (Gaston et al., 2003; Armendáriz et al., 2015). Moreover, an induction of the alternative respiration activity has been described in plants treated with AHAS inhibitors (Gaston et al., 2003; Armendáriz et al., 2015) and gene expression profiling revealed an increased level of alternative oxidase after ABIH treatment (Manabe et al., 2007; Zhu et al., 2008). The induction of these two less efficient ATP-producing pathways

(ethanol fermentation and alternative respiration) has been related to a greater pyruvate availability, since this metabolite is the substrate for PDC and can also act as an allosteric activator of the alternative oxidase.

The increase in pyruvate availability is easily explained to occur as a consequence of AHAS inhibition. However, it was not expected that fermentative induction after GLP treatment would be related to increased pyruvate content, as this herbicide does not inhibit a pyruvate-consuming enzyme. Inhibition of the shikimate pathway at the EPSPS level de-regulates carbon flow into the pathway, causing a massive carbon entrance that accumulates in compounds upstream of the EPSPS inhibition point, such as shikimate (Orcaray et al., 2010). It has been proposed that this shikimate accumulation would divert most of the phosphoenolpyruvate from the glycolytic flow to the shikimate pathway because shikimate is a potent phosphoenolpyruvate carboxylase inhibitor (De María et al., 2006). It is possible that pyruvate accumulation after GLP treatment is a cross-physiological effect induced by increased availability of phosphoenolpyruvate that is not consumed by phosphoenolpyruvate carboxylase.

Pyruvate is a key metabolite in multiple biosynthetic and catabolic cellular pathways, so pyruvate accumulation might be due to multiple bottlenecks. Although no conclusive cause–effect relationship can be drawn, a clear relationship between pyruvate accumulation and fermentation induction can be proposed. What remains to be elucidated is whether pyruvate accumulation is the only cause or a player in a cascade of signals after herbicide treatment. To check whether this metabolite is a key regulator in the induction of fermentation in plants after ABIH treatment, it was studied whether exogenously supplied pyruvate regulates the fermentation in the same way as it is regulated after ABIH application. This study is presented in the Chapter 2 of this thesis.

A decrease in the lactate content in the roots of *A. thaliana* was also observed as a consequence of both herbicide application (Figure 1.37), by

contrast, lactate shown to accumulate in the leaves of GLP-treated plants (Figure 1.36). An induction of LDH has been described in the roots of pea plants treated with imazethapyr (Gaston et al., 2002; Zabalza et al., 2005), however, the activity of LDH did not increase when pea plants were treated with GLP (Orcaray, 2008). This enzyme converts pyruvate to lactate, and back. LDH is known to induce in plants exposed to anaerobiosis (Perata and Alpi, 1993) and moreover, under aerobic conditions an increase in the mRNA levels of *LDH* has been detected in plants exposed to several stresses (drought, cold, wounding) (Dolferus et al., 2008). As it occurs for the ethanol fermentation, in ABIH-treated plants, the activity of LDH could be induced by an increased pyruvate availability, thus, LDH should be acting in the pyruvate to lactate direction. However, a decrease in the lactate content is observed in the roots when plants are treated with ABIH. On the one hand, the decrease in the lactate content in the roots could be due to lactate mobilization to the shoots (where it is shown to accumulate in response to GLP). Lactate synthesized in the roots has been proposed to transport to the shoots by the transpiration (Rivoal and Hanson, 1993). On the other hand, it could also be due to a lactate efflux into the media, indeed *Arabidopsis* has been shown to be able to exude lactate efficiently into the media, preventing its accumulation in the cells to toxic levels (Dolferus et al., 2008).

Another common effect provoked by the application of both herbicides is observed in the leaves regarding the content of the organic acids from the TCA cycle. Both IMX and GLP provoked an accumulation of α-ketoglutarate, succinate and malate, and a decrease in the citrate content in this organ (Figure 1.36). The content of these metabolites was monitored in the roots of imazethapyr-treated pea plants (Zabalza et al., 2011) and different results were obtained. In that study, a general accumulation of glycolytic metabolites upstream of pyruvate and the decrease in several TCA cycle intermediates were observed, suggesting a role for pyruvate controlling respiration during imazethapyr treatment (Zabalza et al., 2011).

Glutathione is a simple sulphur compound composed of three amino acids (cysteine glutamate and glycine); its functions are manifold, but notably include redox-homeostatic buffering (Noctor et al., 2011) and is also important in herbicide detoxification (Dixon and Edwards, 2010). In this study it was evaluated the possible effect of herbicides on its content (Figure 1.39). Both IMX and GLP provoked an increase of total glutathione in the leaves and the roots of wild-type plants. The increase in the total glutathione content could be related to an increase in the availability of its precursors, as cysteine (Figures 1.30 and 1.31), glutamate (Figures 1.28 and 1.29) and glycine (Figures 1.30 and 1.31) contents increased in the IMX- or GLP-treated plants. Both herbicides provoked an accumulation of the reduced form of the glutathione in the two studied organs. By contrast, the oxidized form of glutathione only increased in the leaves after GLP treatment but it did in the roots after both herbicide application. Previous studies shown an accumulation of reduced glutathione in the roots of imazethapyr-treated pea plants (Zabalza et al., 2007). Additionally, studies conducted in GLP-sensitive and resistant soybean demonstrated an increase in the reduced and oxidized glutathione forms in the GLP-sensitive line treated with GLP, while in the resistant line, only the oxidized form was accumulated after GLP treatment (Vivancos et al., 2011).

c) Physiological effects in the *A. thaliana adh1* mutant plants

The physiological effects provoked by IMX and GLP were evaluated in the *A. thaliana adh1* mutants. As expected, no ADH activity was detected in the mutant plants lacking the *ADH1* gene and regarding the activity of PDC, similar response to the wild-type plants was observed in the mutants (Figure 1.22).

As it occurred in the wild-type plants, both shoot and root growth arrest was detected in the *adh1* mutants (Figure 1.23).

Regarding the amino acid content, in the *adh1* mutants the total free amino acids were also accumulated but the increase observed in the roots of this plants as a consequence of IMX or GLP application was slightly less prominent

compared to the accumulation found in wild-type plants (Figure 1.24). As for the individual amino acid content, in general, the same effects were observed in the wild-type and *adh1* mutants, although, some differences were also found. The most remarkable differences are that, contrary to what we observed in wild-type plants, no accumulation of Phe (Figure 1.27) and Cys (Figure 1.30) in the leaves after IMX treatment, of Glu in the leaves and the roots after GLP treatment (Figures 1.28 and 1.29), and of Gln, Asp, Thr and Met in the roots after GLP application were detected in the *adh1* mutants (Figures 1.29 and 1.31). Thus, plants lacking the *ADH1* gene do not show all the effects in the free amino acid content detected in wild-type plants as a consequence of ABIH application.

Effects of IMX or GLP application in the carbohydrate content were also alleviated in the *adh1* mutants (Figures 1.34 and 1.35). In the leaves, contrary to the pattern observed in wild-type plants, GLP did not provoke an accumulation of glucose, sucrose, total soluble sugars and starch. Additionally, the effect of IMX in the total soluble sugars content was slightly alleviated in the *adh1* mutant plants. In the roots of *adh1* mutants, no carbohydrate accumulation was detected, and even, the levels of fructose and glucose were lesser than the ones found in the roots of untreated plants.

As for the organic acids content, the effects of IMX and GLP were slightly alleviated (Figures 1.36 and 1.37). In the leaves of *adh1* mutants, opposite to what occurred in wild-type plants, no pyruvate or lactate accumulation was detected as a consequence of GLP application. Moreover, citrate and α-ketoglutarate did not accumulate in response to IMX. In the roots, contrary to what it was observed for wild-type plants, no changes in the content of pyruvate and lactate were detected after IMX or GLP application.

In the roots of *adh1* mutants, the effect of GLP in the glutathione content was slightly alleviated and no increase in the content of reduced, oxidized and total glutathione was observed (Figure 1.39).

The results obtained for the *adh1* mutants indicate that some of the physiological effects triggered after ABIH application are slightly alleviated in plants lacking the *ADH1* gene, however, both IMX and GLP demonstrated similar toxicity and lethality in the plants of both studied genotypes. Thus, it can be concluded that the lack of the *ADH1* gene does not significantly modify the physiological toxicity or lethality of the ABIH.

Nevertheless, the lack of significant changes in the physiological toxicity or lethality of the herbicides could be due to the activation of alternative pathways to ADH. The acetaldehyde produced by fermentation in plants treated with herbicides cannot be metabolized by the ADH in the *adh1* mutants, however, other pathways may exist in plants to detoxify the accumulated acetaldehyde under these conditions. Indeed, an alternative pathway to ADH has been observed in plants to degrade the acetaldehyde present in the cells. Under different conditions in which the ethanol fermentation is induced (such as, low-oxygen conditions and pollen tube growth), aldehyde dehydrogenases (ALDHs) have been described to metabolize the acetaldehyde and to produce the less toxic compound acetate (Bucher et al., 1995; Tadege and Kuhlemeier, 1997; Tsuji et al., 2000). The existence of this pathway in plants treated with AHAS or EPSPS inhibitors is discussed in the Chapter 3 of the present thesis.

1. 5. CONCLUSIONS

In this chapter the role of ethanol fermentation in the response of the plants to ABIH application has been studied. Two approaches have been done. First, before herbicide application, pea plants were exposed to hypoxia in order to activate the fermentation pathway. IMX effects on this plants were compared with the effects provoked in plants that did not present an induced fermentative metabolism when the herbicide was applied. Second, IMX and GLP effects on *A. thaliana* mutants lacking the *ADH1* gene were compared with the effects provoked by in wild-type plants.

The main conclusions of this chapter are:

- Both type of herbicides provoked the previously described physiological markers in the two studied plant species. As a consequence of AHAS or EPSPS inhibition an induction of ethanol fermentation, a growth arrest and accumulation of soluble sugars, starch and free amino acids were detected. These results validate the use of *A. thaliana* in the study of the effects provoked by ABIH in treated plants.
- Although they inhibit different pathways, both types of herbicides are known to provoke similar physiological effects. In this study, more common physiological consequences for both types of herbicides were found. IMX and GLP provoked a decrease in the citrate content and an increase in the α-ketoglutarate and the malate content in the leaves of treated plants. An increase in the pyruvate levels and a decrease in the lactate levels in the roots of treated plants were also found to be another common effects for both types of herbicides. Moreover, both IMX and GLP provoked an accumulation of glutathione in the leaves and the roots of treated plants.
- The results obtained in this chapter indicate that the role of fermentation in the response of the plants to ABIH application is not the same for all the species. In pea plants, the results show that having the ethanol fermentation induced when the herbicide is applied alleviates the physiological

consequences provoked by the herbicide, indicating that the activation of fermentative pathway reduces the effect of the herbicide. By contrast, in *A. thaliana* some of the effects provoked by ABIH treatment are alleviated when the plants lack the *adh1* gene, suggesting that plants lacking the ethanol fermentation are less affected by the herbicides. Nevertheless, as lethality did not change in either two experimental approaches, the precise role of fermentation cannot be outlined.

- CHAPTER 2 -

Pyruvate and transcriptional regulation of the fermentation pathway in plants treated with herbicides

2. 1. INTRODUCTION

2. 1. 1. Ubiquitin proteasome system

Intracellular protein degradation is necessary to remove either misfolded proteins as well as for the degradation of tagged proteins. In the lysosomes and the plant vacuoles, protein degradation is done in a non-specific manner, while protein degradation in the cytosol and the nucleus is very specific. In eukaryotes, the ubiquitin (Ub)–proteasome system (UPS) is the major pathway for cytosolic and nuclear protein degradation. In this proteolytic pathway, proteins are first modified through covalent conjugation with ubiquitin, which marks them for rapid hydrolysis by the 26S proteasome (Figure 2.1). Ubiquitin is a 76-amino-acid polypeptide found in all eukaryotic species examined. The 26S proteasome is a large, multi-subunit protease found in the cytosol and nucleus, and it is composed by two different complexes (Kurepa and Smalle, 2008). The 20S complex forms a cylinder composed of four heptameric rings and it harbours the proteolytic active site in its interior chamber (Figure 2.1). The 19S complex binds to both ends of the 20S complex and it constitutes the regulatory complex of the 26S proteasome (Figure 2.1).

There are three types of enzymes implicated in the addition of ubiquitin units to the substrate protein: Ub-activating enzymes (E1), Ub-conjugating enzymes (E2), and Ub ligases (E3) (Figure 2.1). Ub conjugation reaction starts with the binding of one Ub to an E1 Ub-activating enzyme. Once activated, the Ub is transferred to an E2 Ub-conjugating enzyme which together with an E3 ligase catalyse the conjugation of the Ub monomer to an internal lysine of the target protein (Figure 2.1). The conjugation of a single Ub unit is not a sufficient signal for degradation, instead, a number of Ub units need to be attached to the target protein (Kurepa and Smalle, 2008).

In plants, the UPS-dependent proteolysis is known to be involved in a multitude of cellular processes, such as, regulation of the cell cycle, regulation of many transcription factors, reticulum-associated protein degradation,

endocytosis of plasma membrane proteins, different stress responses and developmental processes (Ingvardsen and Veierskov, 2001).

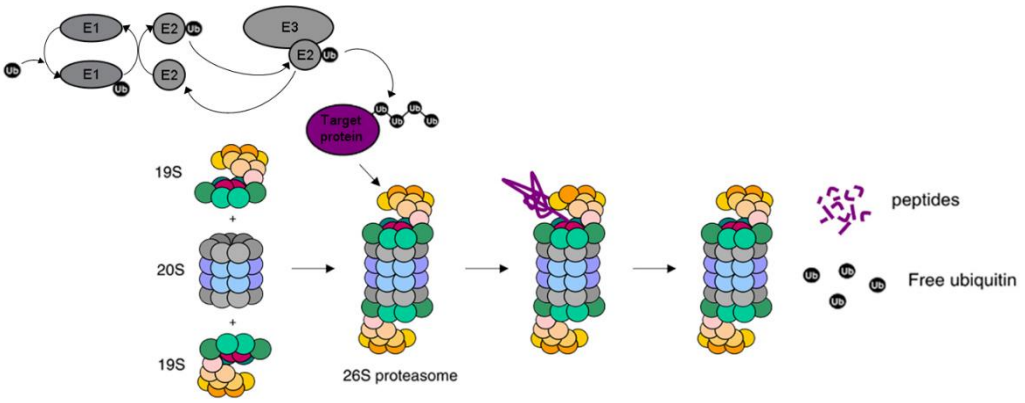


Figure 2.1. Protein ubiquitination and degradation by the 26S proteasome. First, ubiquitin (Ub) is bound and activated by an Ub-activating enzyme (E1), which transfers the Ub to an Ub-conjugating enzyme (E2). This enzyme interacts with the ubiquitin ligase (E3) that specifically recognizes the substrate proteins and mediates the ubiquitin linkage to the target protein. The 26S proteasome is responsible for the degradation of polyubiquitinated proteins, which are cleaved in small inactive peptides. It consists of a 20S core and two 19S lid components bound to both sides of the 20S core protein. The 20S part contains the catalytic domain, which harbours the protease activities and the 19S component recognizes polyubiquitinated proteins and subsequently unfolds and removes ubiquitin from substrates (modified from Marteijn et al., 2006).

2. 1. 2. The N-end rule pathway (NERP) in plants

The NERP is a protein degradation pathway that relates the metabolic stability of a protein to the nature of its N-terminal amino acid residue (Bachmair et al., 1986). This pathway is present in all studied organisms, prokaryotes and eukaryotes, and it is part of the UPS in eukaryotes.

The amino acids present in the N-terminal residue of a protein serve as recognition determinants for protein degradation and are classified as stabilizing or destabilizing residues. The destabilizing residues are known as N-degrons and, in eukaryotes, they can be classified into three hierarchical levels: primary, secondary and tertiary (Figure 2.2) (Varshavsky, 1997). The primary destabilizing residues can be recognized by the E3 Ub ligases (termed N-

recognins) which allows the ubiquitination of the substrate protein for its degradation by the 26S proteasome. The primary destabilizing residues can be subdivided in type 1 (basic residues: Arg, Lys and His) and type 2 (bulky hydrophobic residues: Phe, Trp, Tyr, Leu and Ile) (Figure 2.2). To date, two N-recognins have been found in *Arabidopsis* (named PROTEOLYSIS (PRT)1 and PRT6) which show little similarity (Bachmair et al., 1993; Garzón et al., 2007). While the PRT1 N-recognin recognizes proteins containing aromatic hydrophobic residues in the N-terminal, the PRT6 promotes the degradation of basic N-degrons (Figure 2.2). Other proteins have been propose to act as N-recognins, but their function still remains unclear (Licausi et al., 2013). Unlike primary destabilizing residues, secondary and tertiary destabilizing residues cannot be recognized by the E3 Ub ligases and they need to be modified to generate primary destabilizing residues. Proteins harbouring secondary destabilizing residues (oxidized Cys, Asp and Glu) are arginylated by arginyl-tRNA-transferases (R-transferases) before being recognized by N-recognins and targeted for degradation (Figure 2.2). Two types of R-transferases have been described in *A. thaliana* (named AtATE1 and AtATE2) which have been shown to be functionally redundant (Yoshida et al., 2002). On the other hand, proteins harbouring tertiary destabilizing residues (reduced Cys, Asn and Gln) are first converted into secondary destabilizing residues which are then arginylated to generate primary destabilizing residues (Figure 2.2). Asn and Gln need to be enzymatically deamidated by Nt^{N}- and Nt^{Q}-amidases, respectively, while reduced Cys is oxidized, probably by oxygen and nitric oxide (NO) (Graciet and Wellmer, 2010).

Another pathway for the generation of N-degrons known to occur in yeast and animals is the acetylation of the N-terminal residues by the N-terminal acetylases (Polevoda et al., 2009). Although different candidates of N-terminal acetylases have been proposed in plants the existence of this pathway in plants needs further investigation (Licausi et al., 2013).

After its discovery, the NERP has been examined in different model organisms and was found to be involved in a multitude of cellular and developmental processes. In plants, this pathway is known to participate in leaf senescence, seed germination, shoot and root development and the perception of oxygen availability, however, many physiological processes in which the NERP is involved remain to be discovered (Licausi et al., 2013).

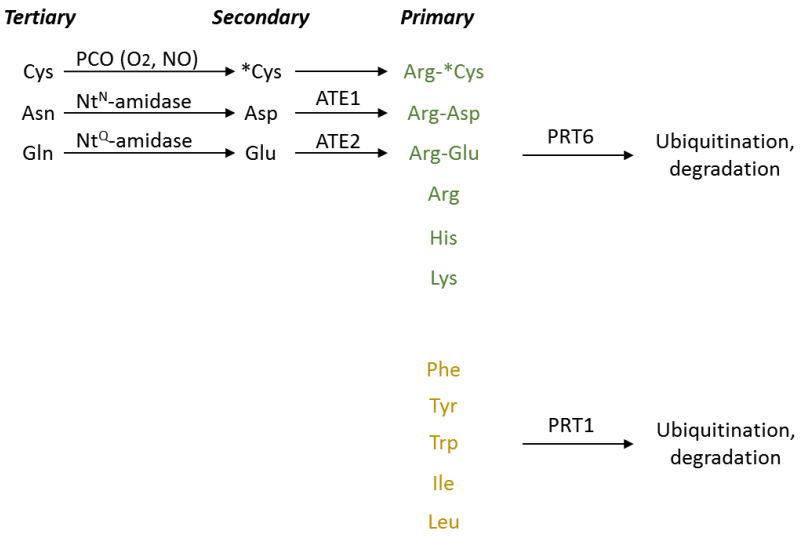


Figure 2.2. The N-end rule pathway in plants. In the nucleus and the cytosol, proteins are degraded by the 26S proteasome. The target proteins are first polyubiquitinated by N-recognins. To date, two N-recognins have been found: PROTEOLYSIS (PRT) 6 and PRT1, which recognize Type 1 (basic N-terminal amino acids, shown in green) and Type 2 (aromatic hydrophobic N-terminal amino acids, shown in yellow), respectively. Type 1 and Type 2 N-terminal residues are Primary destabilizing residues and they are produced from Secondary destabilizing residues. To form Primary destabilizing residues, Secondary destabilizing residues are arginylated by arginyl-tRNA-transferases (ATE1 and ATE2). Secondary destabilizing residues are produced via covalent modification of the Tertiary destabilizing residues. In detail, when Cys is in the N-terminal amino acid, it is oxidized by plant cysteine oxidases (PCOs) (in the presence of O_2 or NO) forming an oxidized Cys (*Cys)). By contrast, in the case of Asn and Gln, this Tertiary destabilizing residues are deamidated by Nt^N- or Nt^Q-amidases, respectively, to form Secondary destabilizing residues (modified from Licausi et al., 2013).

2. 1. 3. Low-oxygen stress in plants

In aerobic organisms, oxygen is the terminal acceptor of electrons in the respiratory chain to produce energy. Moreover, molecular oxygen is involved in a wide variety of chemical reactions in plant metabolism such as the production of reactive oxygen species (ROS), fatty acid desaturation and the synthesis of different phytohormones. Despite its importance, unlike animals, plants lack an efficient oxygen transport mechanism to distribute oxygen to all cells and instead, the supply of oxygen to tissues depends on its passive transport.

When the availability of oxygen becomes limiting, plants can experience oxygen deficiency, which is a rather common phenomenon in plants. Hypoxic conditions have been detected in several plant organs such as fruits, roots and tubers, moreover, plants can be exposed to low oxygen conditions in waterlogged soils and during floods (van Dongen and Licausi, 2015). Under these circumstances, plants have to adapt their metabolism, in order to avoid energy shortage. Different metabolic adaptations have been described in plants exposed to low oxygen conditions which can be rapidly reversed if the oxygen availability increases (Kosmacz and Weits, 2014; van Dongen and Licausi, 2015).

Under limiting oxygen conditions plants cannot produce ATP in the oxidative phosphorylation. To compensate the energy deficiency under that circumstances, glycolytic activity is upregulated to maximize ATP production (Pasteur effect) (Gibbs and Greenway, 2003). To avoid glycolysis become limited by the availability of NAD^+, fermentative pathways are activated. These pathways use pyruvate and NADH as substrates and regenerate the NAD^+ required in the glycolysis (Tadege et al., 1999). The main fermentative pathways induced in plants under low-oxygen conditions have been presented in the section 1.1.2 of the Chapter 1 of the present thesis.

The NO cycle represents another pathway for NAD^+ regeneration under low oxygen conditions. Under hypoxia, NO production from nitrite reduction in a reaction catalysed by nitrate reductase is activated (Limami et al., 2014). Later, the produced NO can be oxidized and produce nitrate by class-1

nonsymbiotic hemoglobins (HB1), indeed, the expression of *HB1* is upregulated when the oxygen becomes limiting (Igamberdiev and Hill, 2004). In this cycle NADH is oxidized and the NAD^+ required for glycolysis is regenerated.

Since ATP production in the glycolysis and NO-cycle is not as efficient as ATP production by the oxidative phosphorylation, when plants are exposed to low oxygen availability the metabolism is adjusted to save ATP (van Dongen and Licausi, 2015).

On the one hand, under these circumstances, non-essential, energy-consuming processes (such as metabolism of starch, protein and lipids) are reduced (Geigenberger, 2003).

Moreover, PPi-dependent reactions are favoured above those which use ATP as substrate (Greenway and Gibbs, 2003). For example, under hypoxic conditions, sucrose is preferentially hydrolysed by sucrose synthase rather than by sucrose invertase. While the phosphorylation of the hexoses produced when sucrose is degraded by invertase requires two ATPs, when sucrose is hydrolysed by sucrose synthase only one UTP and one inorganic pyrophosphate are needed (Bologa et al., 2003).

Another metabolic adaptation of the plants to low-oxygen availability to save energy is the redirection of the TCA cycle to a non-cyclic flux, which reduces $NAD(P)^+$ consumption and NAD(P)H production (Rocha et al., 2010; Sweetlove et al., 2010).

2. 1. 3. 1. Oxygen sensing in plants

The fact that plants need to adapt their metabolism to cope with the reduction in ATP availability when oxygen is limiting highlights the importance of the existence of efficient mechanisms in plants that allow to perceive the oxygen availability in the surroundings.

a) *The N-end rule pathway in low-oxygen conditions*

In plants, the oxygen-sensing pathway is regulated via the oxygen dependent branch of the NERP. The transcription factor (TF) belonging to the group VII of the Ethylene Response Factors (ERF VII) are key activators of the anaerobic response and it has been demonstrated that they contain a highly conserved N-terminal MCGGAI motif which dictates their stability (Gibbs et al., 2011; Licausi et al., 2011a). In the presence of oxygen the ERF VII are degraded by the NERP, while in low-oxygen conditions they are stabilized and can activate the anaerobiotic response (Figure 2.3).

The conserved N-terminal motif MCGGAI is recognized by specific methionine amino peptidases that cleave of the initial methionine and leave the Cys exposed as the N-terminal residue (Bradshaw et al., 1998). The N-terminal Cys is oxidized by plant cysteine oxidases (PCOs) using oxygen as substrate (Weits et al., 2014) which allows its recognition by the R-transferases (ATE1 and ATE2). It has been observed that also NO can oxidize the N-terminal Cys (Gibbs et al., 2014). R-transferases add an Arg to the N-terminus of the ERF VII generating a primary destabilizing residue (Graciet and Wellmer, 2010). Finally, the exposure of an N-terminal Arg is recognized by the E3 ligase PRT6 that polyubiquitinates the target protein and provokes its degradation by the 26S proteasome (Garzón et al., 2007). By contrast, if the oxygen availability decreases, the N-terminal Cys cannot be oxidized and thus, the ERF VII is not degraded and can activate the anaerobiotic response.

In *A. thaliana* there are five TFs belonging to the family ERF VII: RAP2.2, RAP2.3, RAP2.12, HRE1 and HRE2. While the *RAP2.2*, *RAP2.12* and *RAP2.3* TFs are constituvely and ubiquitously expressed, the two other ERF VII TFs, *HRE1* and *HRE2*, are upregulated by hypoxia (Licausi et al., 2010). Other elements (such as darkness, ethylene and abscisic acid) also regulate the transcription of ERF VII TFs (Bailey-Serres et al., 2012; Papdi et al., 2015). Upon hypoxia, the TF RAP2.2 and RAP2.12 have been suggested to trigger the initial anaerobic response, while HRE1 and HRE2 have been suggested to maintain the

anaerobic response along the time (Licausi et al., 2010). The TF RAP2.12 has been found to control the expression of different hypoxia marker genes, such as *PDC1*, *ADH1*, *HB1*, *LBD41*, *HUP7*, *HRA1* and *PCO2* (Licausi et al., 2011a; Weits et al., 2014). The role of RAP2.3 in low-oxygen stress has not been deeply investigated but a recent study demonstrated that this TF acts redundantly with RAP2.2 and RAP2.12 in multiple stress responses, including low-oxygen stress (Papdi et al., 2015).

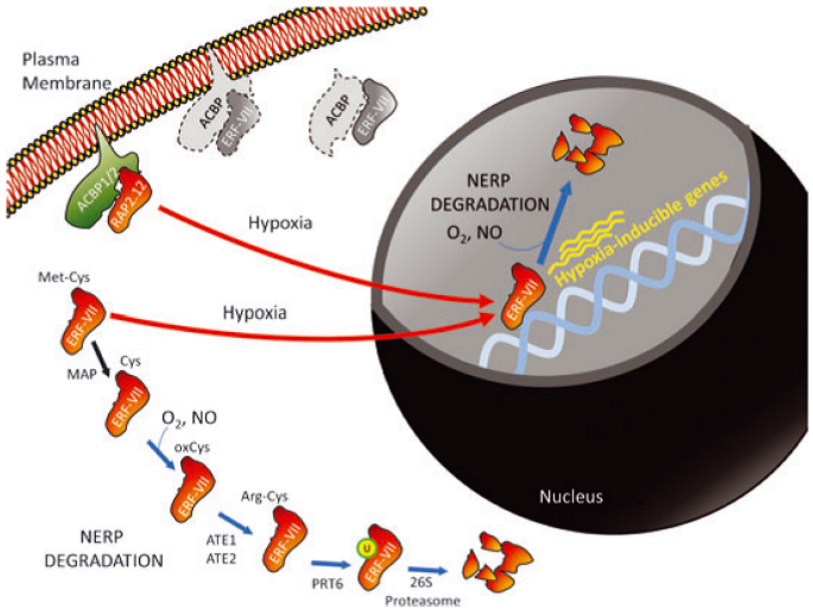


Figure 2.3. Schematic representation of oxygen signaling in plants. The transcription factor RAP2.12 is localized in the plasmatic membrane bound to the acyl-CoA binding protein 1 and 2 (ACBP1 and ACBP2). In grey, the possibility of the interaction of other ERF VII with ACBPs is represented. The methionine present in the N-terminal position of the ERF VII is removed by methionine aminopeptidases (MAPs) and thus Cys is exposed in the N-terminal position. In the presence of oxygen (blue arrows) the ERF VII is degraded by the 26S proteasome via the N-end rule pathway (NERP). In this pathway, the N-terminal Cys is oxidized by plant cysteine oxidases in the presence of oxygen or nitric oxide (NO). The oxidized Cys is recognized by the arginine-transferase 1 and 2 (ATE1 and ATE2) and one Arg residue is bound to the oxidized residue. The N-terminal Arg is recognized by the E3 ligase PROTEOLYSIS6 (PRT6) promoting the polyubiquitination (indicated by yellow U) of the ERF VII and its degradation by the 26S proteasome. Under low-oxygen conditions (red arrows) the N-terminal Cys of the ERF VII is not oxidized and thus, it is not degraded following the NERP. The stable ERF VII moves to the nucleus and activates the anaerobiotic response. Upon reoxygenation, the ERF VII is rapidly degraded following the NERP (Licausi, 2013).

b) *Role of acyl-CoA-binding proteins (ACBPs) in oxygen sensing*

It has been observed that RAP2.12 interacts with ACBP1 and 2 and GFP-fusion experiments demonstrated that under normal oxygen levels RAP2.12 is localized in the plasma membrane whereas under hypoxia it accumulates in the nucleus (Licausi et al., 2011a). A model of the subcellular localization and movement upon low O_2 sensing has been proposed. Under aerobic conditions, RAP2.12 has been suggested to be retained at the plasma membrane bound to ACBP1 and ACBP2 (Figure 2.3) (Licausi et al., 2011a). The association of RAP2.12 to the ACBPs prevents its degradation upon aerobic conditions and it provides a reservoir to quickly induce the anaerobic genes if the oxygen availability decreases, indeed, the induction of the anaerobic genes occurs within 30 min of hypoxia (Licausi et al., 2011b).

By contrast, under low oxygen conditions RAP2.12 is dissociated from the plasma membrane and translocated to the nucleus where it activates the molecular response to oxygen deficiency (Figure 2.3) (Licausi et al., 2011a). How RAP2.12 is released from the membrane and whether it migrates alone or together with ACBPs toward the nucleus still remains to be investigated.

c) *Other possible players in oxygen signalling in plants*

Additional factors have been suggested to regulate the oxygen signaling response. On the one hand, HB1 has been proposed to regulate the oxidation of the N-terminal Cys of the RAP2.12 under low oxygen conditions. Under hypoxia, the levels of NO dramatically increase (Dordas et al., 2003), NO has been suggested to promote RAP2.12 N-terminal Cys oxidation and thus, induce RAP2.12 degradation in *Arabidopsis* (Gibbs et al., 2014). Since HB1 is a NO scavenger, it has been suggested that, under hypoxia, HB1 could reduce the levels of NO and restrict the N-terminal Cys oxidation preventing the proteasomal degradation of RAP2.12, this way HB1 would support the activation of the molecular response to hypoxia (Licausi, 2013). Interestingly, in *Arabidopsis,* HB1 has been shown to be a direct target of RAP2.12 and to be

strongly induced upon hypoxia (Licausi et al., 2011a). However, the precise role of HB1 in the control of the stability of RAP2.12 is still uncertain.

The activity of RAP2.12 has also been found to be modulated by the *HRA1*. The expression of *HRA1* is upregulated by hypoxia, which binds to RAP2.12 and limits its activity (Giuntoli et al., 2014).

Ethylene, whose synthesis is boosted under hypoxia (Jackson, 1985), has also been proposed to regulate oxygen signalling in plants. This hormone has been observed to play a crucial role in flooding tolerance in different plant species, such as some rice varieties, since it enhances internode elongation (Hattori et al., 2009). Additionally, most ERF VIIs (RAP2.2, RAP2.3 and HRE1) are ethylene inducible, although how this regulation affects the oxygen sensing mechanism has not been clarified yet (Licausi, 2013).

Other elements, which are indirectly affected by oxygen availability, have been proposed to play an indirect role in oxygen sensing (Kosmacz and Weits, 2014):

- In low-oxygen conditions, lactic fermentation sustains the initial NAD^+ regeneration, consequently, lactate is accumulated in the cytosol which provokes a decrease in the pH status to an optimal level for PDC activity; this leads to ethanol fermentation activation and lactate fermentation inactivation (Davies et al., 1974).

- Additionally, elevation of cytosolic Ca^{2+} levels upon hypoxia have been shown to be necessary for the induction of *ADH1* and *SUCROSE SYNTHASE 1* gene expression (Subbaiah et al., 1994), indicating that the Ca^{2+} levels indirectly affect the anaerobic response.

- ROS, which are accumulated under hypoxia, have also been shown to play a role in the hypoxic response. Hydrogen peroxide has been observed to be necessary for the expression and activity of ADH under low oxygen conditions (Baxter-Burrell et al., 2002). Moreover, ROS

have also been proposed to regulate N-terminal Cys oxidation and promote ERF VII degradation (Licausi et al., 2013).

- Another key parameter determining the induction of ethanol fermentation is pyruvate concentration. It has been proposed that the different K_m of pyruvate dehydrogenase (PDH) and PDC for pyruvate are the controlling factors that regulate the entry of pyruvate into the TCA cycle or the ethanolic fermentation pathway. The Km of plant PDH's for pyruvate is in the μM range whereas that of PDC's is in the mM range. Under aerobic conditions, pyruvate concentration is very low, and pyruvate preferentially enters the TCA cycle. But when respiration is blocked by inhibitors or by lack of oxygen, pyruvate concentration increases and it becomes available for the PDC reaction (Tadege et al., 1999).

2. 1. 4. Induction of ethanol fermentation upon herbicide treatment

Induction of fermentation is a well-known physiological effect observed in plants upon ABIH application, even if no oxygen availability limitation exists in the surroundings. An increase in the activities of PDC and ADH and their protein contents has been described in plants after AHAS, GS or EPSPS inhibition (the three enzymes blocked by the commercially available herbicides that inhibit amino acid biosynthesis) (Gaston et al., 2002; Zabalza et al., 2005; Zabalza et al., 2011; Orcaray et al., 2012; Armendáriz et al., 2015).

The regulation of the ethanol fermentation has been deeply studied in plants exposed to low-oxygen conditions. By contrast, the regulation of this response in plants upon other stresses, including herbicide treatment, has not been studied yet. AHAS catalyses the condensation of either two molecules of pyruvate to form acetolactate or one molecule of pyruvate with one molecule of 2-ketobutyrate to form 2-aceto-2-hydroxybutyrate (Singh, 1999). Blockage of AHAS-catalysed reactions would result in higher availability of the AHAS substrates, such as pyruvate. So, induction of ethanol fermentation following

AHAS inhibition could be related to an increased availability of pyruvate, since this metabolite is a common substrate for PDC and AHAS. Indeed, ethanol fermentation was induced in the roots of pea plants after exogenous pyruvate application (Zabalza et al., 2009). However, that study revealed that PDC induction cannot simply be explained by increased substrate availability and instead, it was related to a drop in the energy status (Zabalza et al., 2009). One should be careful to conclude that this is the immediate consequence of higher substrate availability for the fermentation pathways only. Fermentation can also be regarded as a general physiological response after a stress situation as has been reported for other abiotic stresses, such as low temperature and osmotic stress (Dolferus et al., 1994; Kürsteiner et al., 2003). These two different explanations are, however, not mutually exclusive and they may even act in concert. Indeed, the analysis of fermentative activity following treatment of herbicides (other than AHAS inhibitors that do not directly affect a pyruvate-consuming enzyme) showed that the roots of pea plants treated with GLP or glufosinate also showed induction of aerobic fermentation (Orcaray et al., 2012; Armendariz et al., 2015).

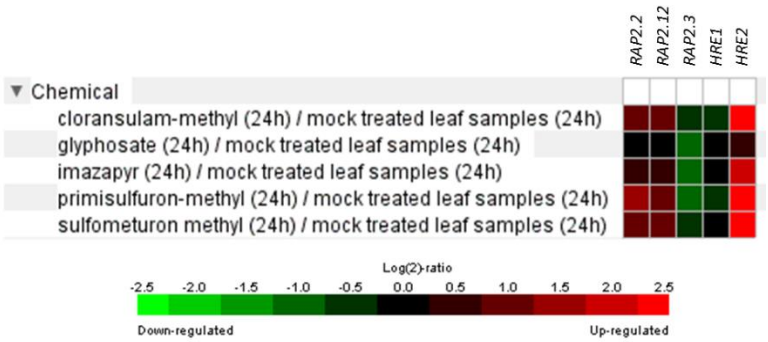


Figure 2.4. The expression (mRNA level) of the five members of the Ethylene Responsive Factors-Group VII in the leaves of herbicide-treated *Arabidopsis thaliana* plants (Hruz et al., 2008).

On the other hand, contrary to the anaerobic fermentation, the induction of fermentation in response to herbicide application is not related to a change in respiratory rates or to a decrease in the energy charge, as the energy charge actually increased (Zabalza et al., 2011; Orcaray et al., 2012).

Nevertheless, several signals known to play a role in the regulation of the fermentative response after anaerobiosis have been found to be affected by ABIH treatment. These results suggest that signals underlying the fermentative response after herbicide treatment might be similar to the fermentative response after low-oxygen stress. The Genevestigator heat map performed with the data from Das et al., 2010 revealed changes in the expression (mRNA levels) of the different ERF VII TFs as a consequence of ABIHs application, being the upregulation of *HRE2* the most remarkable effect (Figure 2.4) (Hruz et al., 2008). Moreover, another study revealed downregulation of RAP2.3 and upregulation of HRE2 after AHAS inhibition (Manabe et al., 2007). These results suggest a possible role of the ERF VII TFs in the induction of the ethanol fermentation upon herbicide treatment. Moreover, several studies have reported that the 26S proteasome is involved in the physiological response to ABIHs (Kurepa et al., 2010; Zulet et al., 2013a). It can be hypothesized that if there is a common signalling pathway between the induction of fermentation after anaerobiosis and after ABIH treatment, it would be very interesting to check the role of the RAP2.12 after herbicide treatment and its possible interplay with pyruvate availability.

2. 2. OBJECTIVES

Plants possess efficient mechanisms to perceive the oxygen availability in the surroundings. In plants, the oxygen-sensing pathway is regulated via the oxygen dependent branch of the NERP and a cascade of regulatory mechanisms interplay in the metabolic response to low-oxygen. Transcriptional regulation of ethanol fermentation in plants under low-oxygen conditions has been deeply studied and it has been established that the TF RAP2.12 regulates the *PDC* and *ADH* gene expression. The stability of RAP2.12 is regulated by the NERP, while this TF is degraded under normal oxygen conditions, it is stabilized upon hypoxia. Additional factors have been suggested to regulate the oxygen signalling response, such as the pyruvate concentration.

The importance of the induction of the fermentation pathway in the toxicity of plants treated with ABIHs has been discussed in the First Chapter. By contrast, little is known about the regulation of the induction of this pathway in the plants exposed to ABIHs.

The main objective of the Chapter 2 of this thesis is **to evaluate the regulation of the ethanol fermentation in plants after AHAS or EPSPS inhibition.** This general aim was approached by these specific objectives:

1. To evaluate whether the ethanol fermentation is regulated at a transcriptional level in plants after AHAS or EPSPS inhibition. For that purpose, the transcript levels of the *PDC* and *ADH* in pea and *A. thaliana* plants treated with ABIHs were evaluated.
2. To analyse the possible role of pyruvate as a signal in the regulation of ethanol fermentation, checking if an increase in the pyruvate availability, the substrate of the PDC, induces the ethanol fermentation pathway. For that purpose, the possible induction of the ethanol fermentation after pyruvate supply to plants grown under axenic conditions was evaluated and compared with ABIH treatment.

3. To evaluate if, as it occurs in low-oxygen stress, the TF RAP2.12 is involved in the induction of *PDC* and *ADH* upon herbicide or pyruvate treatment. For that purpose, the expression level of different RAP2.12-regulated genes were evaluated in *A. thaliana* seedlings grown in axenic plates and supplied with ABIHs or pyruvate. Moreover, the effect of herbicides and pyruvate on the NERP-mediated proteolysis was also evaluated.

2. 3. MATERIALS AND METHODS

2. 3. 1. Plant material and treatment application

2. 3. 1. 1. *Pisum sativum*

a) *Hydroponic system*

Pea plants were grown in a hydroponic system as described in the section 1.3.1.a of the Chapter 1 of the present thesis.

The treatments were applied when the plants were 12-days-old. Plants were separated in three groups. Two of the groups were treated with herbicides and the third group of the plants was not treated and was the control group for the herbicide-treated plants. IMX or GLP were applied to the nutrient solution as commercial formulations at a final concentration of 5 mg active ingredient L^{-1} (16.33 µM) of IMX (Pulsar®40, BASF Española SA, Barcelona, Spain) or 53 mg active ingredient L^{-1} (232.27 µM) of GLP (Glyfos®, Bayer CropScience, S.L, Paterna, Valencia, Spain). The experiment was performed in triplicate.

For the analytical measurements, intact root samples were taken at days 1, 3 and 7 after herbicide application. Plant material was immediately frozen in liquid nitrogen and stored at -80°C for further analysis. Later, frozen samples were ground under liquid nitrogen using a Retsch mixer mill (MM200, Retsch®, Haan, Germany), the needed amount of tissue for each analysis was separated and stored at -80°C.

b) *Axenic system*

Since pyruvate can be easily degraded by bacteria, another set of plants was grown in an axenic system. The seeds were sterilized as described in section 1.3.1.a of the Chapter 1 of the present thesis, but all the reactives were first sterilized in an autoclave or by filtering with a 0.22 µm filter.

The seeds were sown in magenta boxes filled with water, they were placed in a mesh to maintain them in contact with the water (and later with the nutrient solution) but to avoid submergence (Figure 2.5). The bottom of the boxes was

covered to reduce the exposure of the roots to the light (Figure 2.5). To avoid contamination, all the system was sterilized before sowing the seeds. The boxes were placed in a growing chamber with the following growing conditions: 120-150 μmol m^{-2} s^{-1} light, at 23°C/18°C day/night temperature and at 12/12h day/night cycle photoperiod.

Four days later, the water was replaced with sterile nutrient solution described in Rigaud and Puppo (1975) with minor modifications (detailed in the section 1.3.1.a of the Chapter 1 of this thesis). To prevent roots from hypoxia the nutrient solution was continuously aerated and to avoid contamination, before introducing the air into the solution it was filtered with a 0.22 μm gas filter (Figure 2.5).

Treatments were applied when the plants were 6 days-old. At this time point, the nutrient solution was renewed. For the herbicide treatments, IMX or GLP were added to the nutrient solution. The two herbicides were applied as commercial formulations at a final concentration of 5 mg active ingredient L^{-1} (16.33 μM) of IMX (Pulsar®40, BASF Española SA, Barcelona, Spain) or 53 mg active ingredient L^{-1} (232.27 μM) of GLP (Glyfos®, Bayer CropScience, S.L, Paterna, Valencia, Spain). Other plants were treated with 10 mM Na-pyruvate (Sigma-Aldrich Co., St. Louis, MO, USA) added to the nutrient solution. Other plants were exposed to low-oxygen conditions, for that purpose, aeration was removed and the nutrient solution was bubbled with filtered N_2 gas for 5 min every 12 h until the end of the experiment (three days). Another set of plants was not treated and was the control for the treated plants. To avoid contamination, both herbicides and the pyruvate were filtered (with a 0.22 μm filter) before being added to the nutrient solution. All the manipulations were performed under a horizontal laminar flow cabinet and all the material was sterilized before being used.

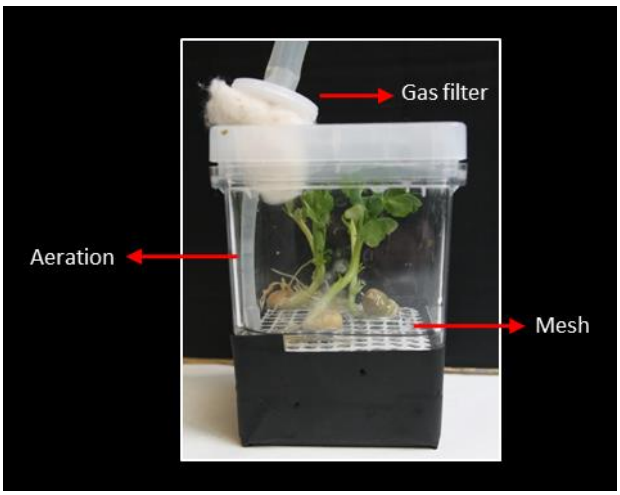


Figure 2.5. Pea plants growing under sterile hydroponic system, with the nutrient solution continuously aerated.

For the analytical measurements, intact root samples were taken at day 3 after the application of the treatments. Plant material was immediately frozen in liquid nitrogen and stored at -80°C for further analysis. Later, frozen samples were ground under liquid nitrogen using a Retsch mixer mill (MM200, Retsch®, Haan, Germany), the needed amount of tissue for each analysis was separated and stored at -80°C.

2. 3. 1. 2. *Arabidopsis thaliana:*

a) *Hydroponic system*

Wild-type *A. thaliana* Col-0 plants were hydroponically grown as described in the section 1.3.1.b.1 of the Chapter 1 of this thesis. An additional treatment was applied which consisted of the exposure of the plants to low-oxygen conditions. For that purpose, the aeration was removed and the nutrient solution was bubbled with N_2 gas for 5 min every 12 h until the end of the experiment (three days).

b) *Axenic system*

Wild-type *A. thaliana* Col-0 and transgenic *A. thaliana* plants containing artificial N-end substrate β-glucuronidase (GUS) reporter constructs (M-GUS or

R-GUS reporter constructs) (kindly provided by Francesco Licausi (PlantLab, Scuola Superiore Sant'Anna, Pisa, Italy)) were sterilized as described in the section 1.3.1.b.1 of the Chapter 1 of this thesis. To ensure axenic conditions all the material and reactives were sterilized before use.

Plants were grown in sterile six-well plates in liquid half-strength MS medium (pH 5.7) (Sigma-Aldrich Co., St. Louis, MO, USA) enriched with 1% (w/v) sucrose, under continuous shaking conditions. Ten seeds were placed in each well and plates were incubated for 3 days at 4°C in darkness for stratification. Plates were then placed in a growing chamber and seedlings were grown under 80 μmol m^{-2} s^{-1} light, at 23°C/18°C day/night temperature and at a 12/12 h day/night photoperiod.

When plants were six days-old, the old growth medium was removed and substituted with fresh one not supplemented with sucrose, and treatments were applied. Seedlings were treated for 5 days with IMX, GLP or pyruvate. The two herbicides were applied as commercial formulations at a final concentration of 1.5 mg active ingredient L^{-1} (4.9 μM) of IMX (Pulsar®40, BASF Española SA, Barcelona, Spain) or 20 mg active ingredient L^{-1} (87.65 μM) of GLP (Glyfos®, Bayer CropScience, S.L, Paterna, Valencia, Spain). Other plants were treated with 10 mM Na-pyruvate (Sigma-Aldrich Co., St. Louis, MO, USA). To avoid contamination, both herbicides and the pyruvate were filtered (with a 0.22 μm filter) before being added to the nutrient solution. Seedlings from one individual well were collected as a biological sample and different wells were harvested as replicates. The plant material was immediately frozen, and fresh material was used for the GUS staining.

2. 3. 2. Pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) activities and soluble protein content

The *in vitro* activities of PDC and ADH were monitored as described in section 1.3.3 of the Chapter 1 of this thesis.

2. 3. 3. The SDS-PAGE and immunodetection of PDC and ADH

Immunodetection of PDC and ADH was monitored as described in the section 1.3.5 of the Chapter 1 of this thesis.

For the detection of PDC and ADH in *A. thaliana*, the antibodies were obtained from Agrisera (Agrisera, Vännäs, Sweden). In the case of PDC, the antibody was diluted to 1:10,000 and the blot was incubated for 1 h at room temperature. For the detection of ADH the antibody was diluted to 1:3,000 and the blot was incubated for 3 h at room temperature. Goat Anti-Rabbit IgG HRP Agrisera (Agrisera, Vännäs, Sweden) was used as the secondary antibody at a dilution of 1:20,000, blot was incubated for 1 h at room temperature and bands were visualized using Amersham™ ECL™ Prime Western Blotting Reagents (GE Healthcare, Buckinghamshire, UK) and a Bio-Rad ChemiDoc Imaging system (ChemiDoc, Bio-Rad Inc., Hercules, CA, USA).

2. 3. 4. Quantitative Real-Time-Polymerase Chain Reactions (qPCRs)

Total RNA was extracted from ground frozen seedlings or root samples (about 0.1 mg FW). Samples were homogenized in 600 µL extraction buffer (50 mM Tris-HCl (pH 8.0), 300 mM NaCl, 5 mM EDTA, 2% SDS) supplemented with 7.5 µl of β-mercaptoethanol and 2.5 µL antifoam. To each sample, 84 µL of 3 M KCl were added and then kept on ice for 15 min. Samples were centrifuged at 7,500 *g* for 5 min at 4°C and the supernatant was transferred to a new tube. To each tube, 600 µL of 8 M LiCl were added and they were incubated 2 h at 4°C. Samples were centrifuged at 21,000 *g* for 20 min at 4°C, the supernatant was discarded and the pellet was resuspended in 400 µL of nuclease-free water. An equal volume (400 µL) of phenol:chloroform:isoamyl alcohol (25:24:1) mixture was added and samples were centrifuged at 21,000 *g* for 2 min at room temperature. The aqueous phase was recovered and an equal volume of chloroform was added and mixed. Samples were centrifuged at 21,000 *g* for 2 min at 4°C. Again, the aqueous phase was recovered and 40 µL of 3 M Na-acetate and 2.5 volumes (1mL) of ice-cold absolute ethanol were added. The samples were kept for 30 min at -20°C for RNA precipitation. Tubes were centrifuged

for at 21,000 *g* for 10 min at 4°C, ethanol was removed and pellet was air dried. The dry pellet was resuspended in 40 µL of nuclease-free water.

Total RNA was subjected to a DNAse treatment using the RQ1-DNase kit (Promega Biotech Ibérica, SL., Alcobendas, Spain). Five hundred ng RNA were reverse transcribed into cDNA using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories Inc., Hercules, CA, USA) following the manufacturer's instructions.

Arabidopsis thaliana

	FORWARD	REVERSE
PDC1 (At4g33070)	cgattatggcactaaccggatt	tgttcaccaccgcctgataac
PDC2 (At5g54960)	acggatcaattggctggtcagtg	atcacacgcctgtttggcatgg
ADH1 (At1g77120)	tattcgatgcaaagctgctgtg	cgaacttcgtgtttctgcggt
HB1 At2g16060	tttgaggtggccaagtatgca	tgatcataagcctgaccccaa
HRA1 (At3g10040)	gggaagaagcggcaagtgtagtg	tttactgcctaatgtcactaaaacgtgag
HUP7 (At1g43800)	accaatgttggcaacccgcttc	tttccctcagctcacgaacctg
LBD41 (At3g02550)	tgaagcgcaagctaacgca	atcccaggacgaaggtgattg
PCO2 (At5g39890)	caccatgggaactgatacagttat	tcattctttgatggttggtcc
ACTIN2 (At3g18780)	tcttccgctctttctttccaagc	accattgtcacacacgattggttg

Pisum sativum

	FORWARD	REVERSE
PDC1 (Z66543)	ggactataccggctttgtgagtgc	accttcgcagtccagcatttcc
PDC2 (Z66544)	atgcacaagcggtacccgag	tttctggccacatcgcagca
ADH1 (X06281)	atggcaactacaagccccgc	agctccagctccccttcat
ß-TUBULIN3 (X54846)	ttgggcgaaaggacactatactg	caacatcgaggaccgagtca

Table 2.1. The list of primers used in the qPCRs.

The qPCR amplification was carried out with the ABI Prism 7300 sequence detection system (Applied Biosystems, Life Technologies, Darmstadt, Germany] using the iQ™ SYBR® Green Supermix (Bio-Rad Laboratories Inc., Hercules, CA, USA). Each reaction was performed for 1 µL of cDNA in a total volume of 10 µL containing: 5 µL of the iQ™ SYBR® Green Supermix (Bio-Rad Laboratories Inc., Hercules, CA, USA), 0.5 µM specific forward primer and 0.5 µM specific

reverse primer. The parameters of the PCR programme were as follows: 10 s 50°C, 3 min 95°C, 40 cycles (15 s 95°C, 30 s 60°C) and a dissociation curve (15 s 95°C, 30 s 60°C and 15 s 95°C). *ACTIN2* (At3g18780) was used as the reference gene for *A. thaliana* and β-*TUBULIN3* (X54846) for pea (Saha and Vandemark, 2012). The primer pairs used in the qPCRs are presented in Table 2.1. Relative quantification of the expression of each individual gene was performed using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001).

2. 3. 5. Histochemical detection of GUS activity

Histochemical GUS staining was carried out according to Jefferson et al., 1987. Plant material was fixed immediately after sampling in cold 90% acetone for 1 h on ice, the medium was frequently changed. Fixed material was rinsed several times in 100 mM phosphate buffer (pH 7), and then incubated in the dark at 37°C for 4 h in a freshly prepared staining solution (10 mM EDTA, 0.1% (v/v) Triton X-100, 0.5 mM $K_3(CN)_6$, 0.5 mM $K_4(CN)_6$ $3H_2O$ and 0.5 mM X-Gluc (previously dissolved in DMSO) in 100 mM phosphate buffer (pH 7)). To eliminate the chlorophyll from green tissues, seedlings were incubated in absolute ethanol until the tissue was destained.

2. 3. 6. Statistical Analysis

The data obtained from this study were analysed by the IBM SPSS Statistics (v.22). The mean was used as a measure of central tendency and the SE as a measure of dispersion. When only two treatments were applied, the data were compared with the Student's *t*-Test. When more than two treatments were applied the data of the treated and non-treated plants were compared with the one-way ANOVA test. The homoscedasticity of variances was confirmed by the Levene's test and the HSD Tukey and Dunnett T3 *post hoc* statistical tests were applied to the homogeneity and non-homogeneity of variances cases, respectively. In all cases, a significance level of 5% ($p < 0.05$) was applied.

2. 4. RESULTS AND DISCUSSION

2. 4. 1. Ethanol fermentation in ABIH-treated plants is transcriptionally regulated

The activity of the enzymes involved in the ethanol fermentation was monitored in pea and *A. thaliana* treated with ABIHs.

The *in vitro* activities of PDC and ADH were monitored in the roots of pea plants treated with IMX or GLP at days 3 and 7 after herbicide treatment (Figure 2.6) and in the leaves and roots of *A. thaliana* Col-0 plants 3 days after the application of the herbicides (Figure 2.7).

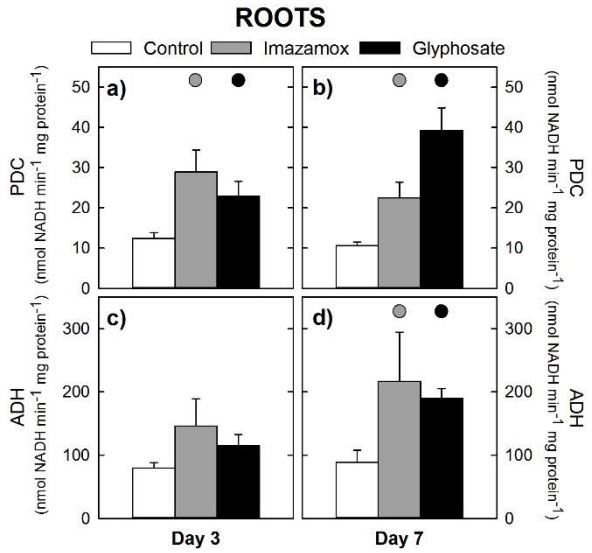


Figure 2.6. The *in vitro* activities of pyruvate decarboxylase (PDC) (a and b) and alcohol dehydrogenase (ADH) (c and d) in the roots of untreated (control) or treated with imazamox or glyphosate pea plants (for 3 and 7 days). Values represent the mean ± SE (n = 5 biological replicates). Significant variations at a given day are marked with ● for differences between control and imazamox-treated plants, and with ● for differences between control and glyphosate-treated plants (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).

It was detected an increase in both activities, in both species and after both herbicides. In pea plants, the activity of PDC significantly increased as a consequence of both herbicide applications at days 3 and 7 after treatment. However, the increase in the activity of ADH was significant only at day 7 after IMX or GLP application.

In *A. thaliana* both herbicides increased the *in vitro* activity of PDC and ADH in the roots and the leaves of herbicide-treated (for 3 days) plants (Figure 2.7).

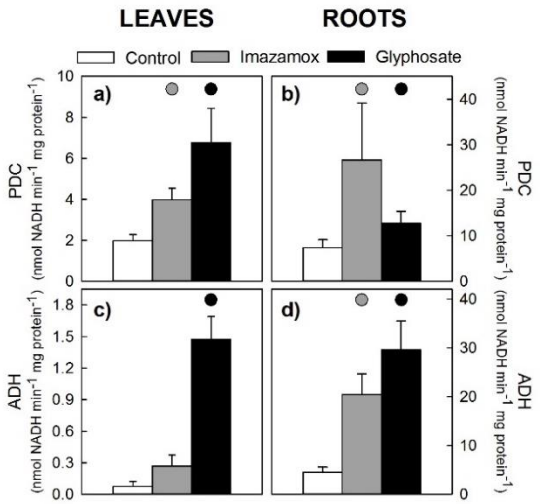


Figure 2.7. The *in vitro* activities of pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) in the leaves (a and c) and the roots (b and d) of wild-type *Arabidopsis thaliana* Col-0 plants, untreated (control) or treated with imazamox or glyphosate for 3 days. Values represent the mean ± SE (n = 5 biological replicates). Significant variations are marked with ○ for differences between control and imazamox-treated plants, and with ● for differences between control and glyphosate-treated plants (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).

The *in vitro* activities of PDC and ADH have been described to be induced in plants treated with ABIHs. (Gaston et al., 2002; Zabalza et al., 2005; Orcaray et al., 2012; Zulet et al., 2013a). Ethanol fermentation has been deeply studied in the response of the plants to low-oxygen conditions. While the transcriptional regulation of the induction of fermentation in plants exposed to low-oxygen conditions has been widely studied, whether the induction of fermentation upon herbicide treatment is transcriptionally regulated has not been studied yet.

Additionally, it was checked if the increase in the activity of PDC and ADH was related to an increase in the enzyme amount. The protein content of PDC and ADH was monitored in the roots of pea plants untreated and treated with

IMX or GLP at days 3 and 7 after herbicide application (Figure 2.8), and in the roots of *A. thaliana* plants 3 days after herbicide application (Figure 2.9).

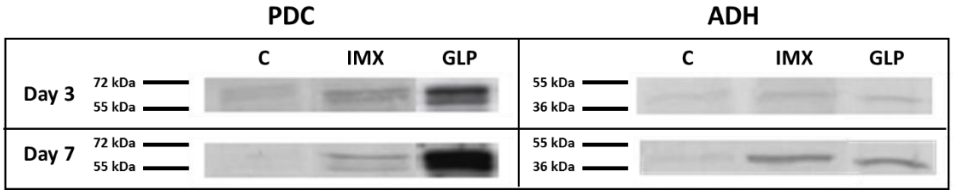


Figure 2.8. Immunoblot detection of pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) in the roots of untreated (control, C) or treated with imazamox (IMX) or glyphosate (GLP) pea plants. The protein contents of PDC and ADH was monitored at days 3 and 7 after herbicide application.

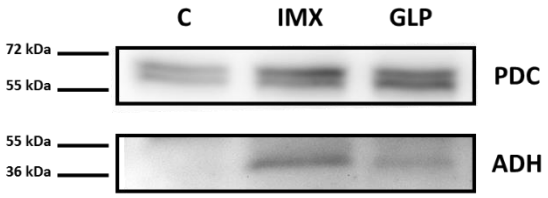


Figure 2.9. Immunoblot detection of pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) in the roots of untreated (control, C) or treated with imazamox (IMX) or glyphosate (GLP) *Arabidopsis thaliana* plants treated for 3 days.

Protein blotting showed that the increases in PDC and ADH activities correlated with the increases in the amounts of the respective proteins for both pea roots (Figure 2.8) and *A. thaliana* roots (Figure 2.9).

The increased free amino acid pool detected after AHAS and EPSPS inhibition has been proposed to be derived from an increase in protein turnover, and a common pattern of proteolysis has been proposed (Zulet et al., 2013a). Indeed, it was shown that, although protein synthesis occurs after AHAS or EPSPS inhibition, the amino acids that comprise these proteins do not contain newly incorporated nitrogen; instead they contain nitrogen that is mainly scavenged from protein degradation (Zabalza et al., 2006). It is notable that plants treated with both classes of herbicides had increased levels of PDC and ADH protein synthesis in a situation in which amino acids have to be scavenged

from existing proteins. This re-use of existing proteins in the synthesis of fermentative enzymes suggests that fermentation plays an important role in the response of the plant to both types of herbicides and therefore, in their toxic physiological effects.

Therefore, the induction of the catalytic activity of the PDC and ADH is an effect of an increase in the protein amount. Nevertheless, it remained to be evaluated if the accumulation of the proteins was due to an increase in their transcript levels.

The two enzymes of the ethanol fermentation pathway, PDC and ADH, were evaluated by relative expression levels of transcripts. In pea roots, the expression of two *PDC* genes (*PDC1* and *PDC2*) and *ADH1* was evaluated after 1, 3 and 7 days of treatment. Both herbicides produced a significant accumulation of the transcripts of all enzymes after 3 and 7 days (Figure 2.10).

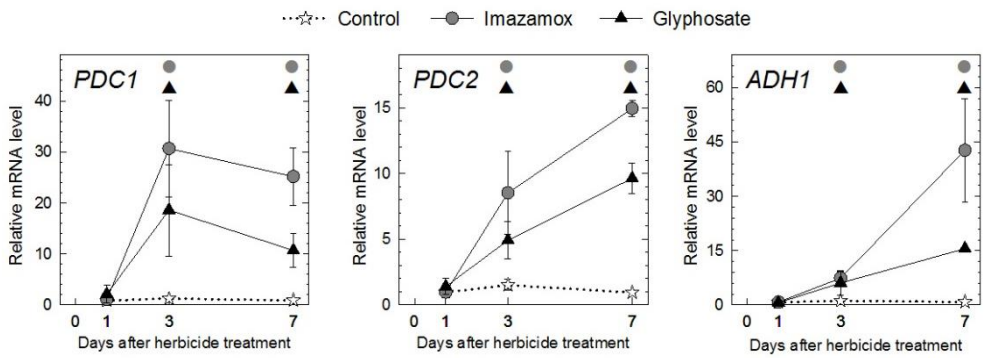


Figure 2.10. Relative transcript levels of the genes *PDC1, PDC2* and *ADH1* in the roots of untreated (control) or treated with imazamox or glyphosate pea plants (for 3 days). Values represent the mean ± SE (n = 4 biological replicates). Significant variations at a given day are marked with ● for differences between control and imazamox-treated plants, and with ▲ for differences between control and glyphosate-treated plants (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).

The expression levels (relative mRNA levels) of two (*PDC1* and *PDC2*) of the four genes belonging to the PDC gene family in *Arabidopsis* (Kürsteiner et al., 2003) and *ADH1* were analysed in the roots of *A. thaliana* three days after treatment with IMX or GLP (Figure 2.11).

To evaluate whether the treated plants experienced a low-oxygen stress, the relative transcript levels of the *HB1* was quantified in ABIH-treated plants and compared with the *HB1* relative transcript levels present in the plants exposed to low-oxygen stress. The transcription level of *HB1* is typically used as a marker of low-oxygen conditions in the plants (Licausi et al., 2010).

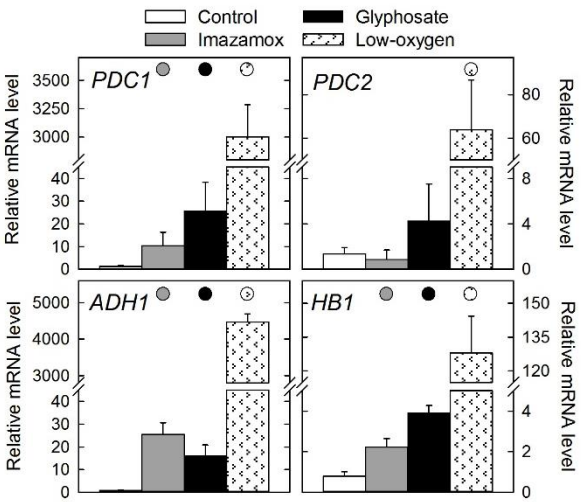


Figure 2.11. Relative transcript levels of the genes *PDC1*, *PDC2*, *ADH1* and *HB1* in the roots of *Arabidopsis thaliana* Col-0 plants, untreated (control), treated with imazamox or glyphosate for 3 days or without aeration for 3 days. Values represent the mean ± SE (n = 5 biological replicates). Significant variations are marked with ● for differences between control and imazamox-treated plants, and with ● for differences between control and glyphosate-treated plants, and ◌ for differences between control and plants exposed to low-oxygen conditions (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).

The transcript levels of the genes involved in the ethanol fermentation were highly increased upon low-oxygen stress, and the transcription of *HB1* was upregulated too. In comparison, the induction of the transcription of the genes

involved in the ethanol fermentation after herbicide treatment was milder, but a significant increase in the relative transcript levels of *PDC1* and *ADH1* could still be detected. Additionally, although the plants were well-aerated throughout the experiment (oxygen at 100% air saturation), the relative transcript levels of the hypoxia marker *HB1* also increased upon ABIH treatment suggesting a similar regulation of fermentation after ABIH application and low-oxygen stress.

These results confirm that the induction of ethanol fermentation in the response of the plants to herbicide application is transcriptionally regulated, since *PDC1* and *ADH1* mRNA levels in the roots of both studied species and *PDC2* mRNA level in the roots of pea plants were upregulated.

2. 4. 2. The possible role of pyruvate in the induction of fermentation

The induction of fermentation after AHAS inhibition can be associated with an increase in the pyruvate availability since this metabolite is a common substrate for both AHAS and PDC. By contrast, induction of fermentation after EPSPS inhibition cannot be easily explained by an increase in the pyruvate availability since EPSPS is not a directly pyruvate-consuming enzyme.

Although induction of fermentation was reported following feeding pyruvate (Zabalza et al., 2009), that study revealed that PDC induction cannot simply be explained by increased substrate availability. Feeding pyruvate to the roots led to an increase of the oxygen consumption rate after 24 h, which ultimately led to anoxia. That said, fermentative metabolism was only activated one day later, when the energy charge of the tissue was decreased, indicating that alcohol fermentation appeared to be primarily induced by a drop in the energy status of the tissue rather than by a low oxygen concentration (Zabalza et al., 2009). Comparison of hypoxia and feeding pyruvate with AHAS inhibition by imazethapyr revealed that while hypoxic roots did not show internal anoxia, feeding pyruvate or applying imazethapyr to the roots led to internal anoxia, probably related to the respiratory burst detected. The three treatments induced ethanol fermentation, but fermentation induced following herbicide treatment

was earlier than that following pyruvate supply and was not associated with a decrease in the energy status (Zabalza et al., 2011).

The organic acid pyruvate is a central metabolite than can be fuelled in many pathways and can be easily consumed by microorganisms present in the surroundings. In this part of this chapter, pea plants were grown in axenic liquid media to prevent contamination. The originality of the study lies in the evaluation whether the response of the plants to both herbicides is related to an increase in the pyruvate availability has been performed under sterile conditions. Pyruvate was supplied to the nutrient solution and the response to this treatment was compared with the response of the plants to IMX or GLP application (to the nutrient solution). As in the previous part of this chapter, low-oxygen stress was added as an extra treatment in this experiment, and it was used as a fermentation induction marker.

The *in vitro* activities of PDC and ADH were measured in the roots of pea plants untreated or treated with pyruvate, IMX or GLP for three days (Figure 2.12). No increase in the activity of PDC was detected after the application of the different treatments, however, the activity of ADH increased as a consequence of the three treatments. The low-oxygen treatment provoked an increase of the *in vitro* activities of PDC and ADH in the roots, showing that it was possible to detect increases of both activities after one treatment, while herbicides only induced the activity of ADH.

To evaluate whether the protein contents of PDC and ADH increased after pyruvate, IMX or GLP application, immunoblot analysis were carried out in the roots of pea plants treated for three days (Figure 2.13). The protein levels of PDC increased as a consequence of pyruvate, IMX or GLP application, while the ADH protein content decreased after pyruvate application and it was not modified as a consequence of IMX or GLP application. The protein content of PDC and ADH also increased in the plants exposed to low-oxygen conditions.

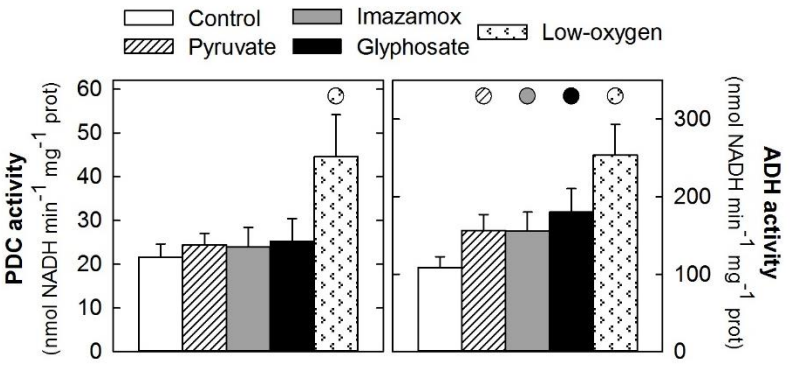


Figure 2.12. Enzymatic activities of pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) in pea roots untreated (control) or roots treated with pyruvate, imazamox or glyphosate, or grown under low-oxygen conditions. Plants were grown in sterile conditions and the treatments were applied for 3 days of treatment). Mean ± SE (n=8). Significant variations are marked with ⊘ for differences between control and pyruvate-treated plants, with ● for differences between control and imazamox-treated plants, with ● for differences between control and glyphosate-treated plants and with ⊘ for differences between control and plants exposed to low-oxygen conditions (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).

An increase in the activities of PDC and ADH and their protein contents has been described in the roots of pea plants treated with pyruvate and after AHAS or EPSPS inhibition (Zabalza et al., 2009; Zabalza et al., 2011; Orcaray et al., 2012). Fermentative induction after GLP treatment is more difficult to relate directly to increased pyruvate content, as this herbicide does not inhibit a pyruvate-consuming enzyme. The inhibition of the shikimate pathway at the EPSPS level deregulates the carbon flow into the pathway, causing a massive carbon entrance that accumulates in compounds upstream of the EPSPS inhibition point, such as shikimate (Orcaray et al., 2010). It has been proposed that this shikimate accumulation would divert most of the PEP from the glycolytic flow to the shikimate pathway because shikimate is a potent PEP carboxylase inhibitor (De María et al., 2006). It can be proposed a potential higher pyruvate availability after GLP treatment as a cross-physiological effect that is induced by increased availability of PEP that is not being consumed by PEP carboxylase.

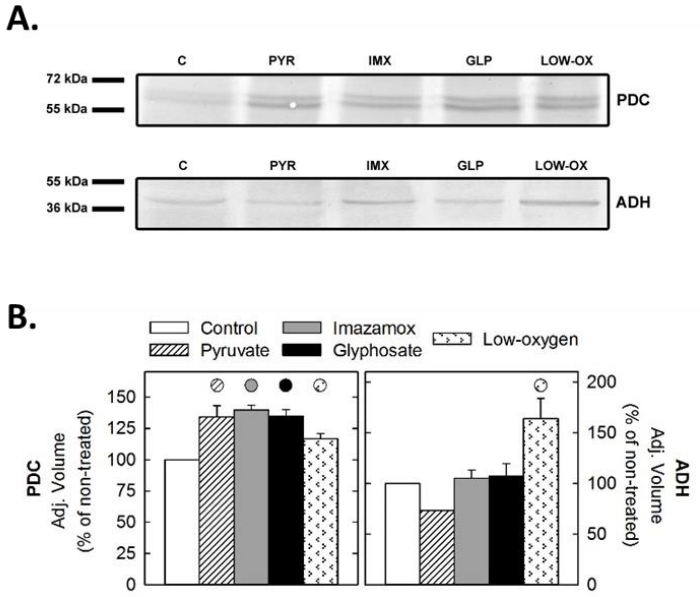


Figure 2.13. Immunoblot detection of pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) in pea roots untreated (control, C) or roots treated with pyruvate (PYR), imazamox (IMX) or glyphosate (GLP) or exposed to low-oxygen conditions (LOW-OX). Plants were grown in sterile conditions and they were harvest after three days of the application of the treatments. Each lane contains 30 µg of protein. **A)** Protein-blots for PDC and ADH, for each treatment one representative sample is shown. **B)** Analyses of band intensity on blots presented as the relative ratio of the control. Control is arbitrarily presented as 100%. Mean ± SE (n=4). Significant variations are marked with ⊘ for differences between control and pyruvate-treated plants, with ● for differences between C and IMX-treated plants, with ● for differences between C and GLP-treated plants, and with ⊙ for differences between C and plants exposed to low-oxygen conditions (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).

Our results confirm that ethanol fermentation is induced after the application of pyruvate, IMX or GLP but the induction was not equivalent in both enzymes. For the three treatments, while an increase in PDC was detected at the protein level, ADH activity *in vitro* was increased. The observed induction was not as significant as the previous reported ones. Previous studies with different doses or at different time points would have been helpful in stablishing the adequate dose and evaluation time point for a maximum induction under these growing conditions.

To analyse whether the induction of fermentation after pyruvate, IMX or GLP application was transcriptionally regulated, the transcript levels of the genes *PDC1*, *PDC2* and *ADH1* were measured by qPCR in the roots of pea plants (Figure 2.14).

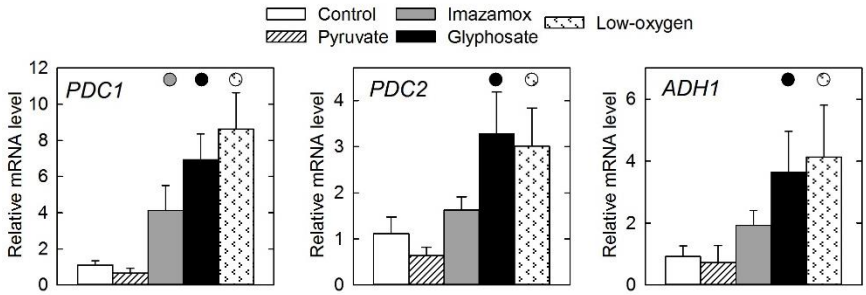


Figure 2.14. Relative transcript levels of the genes *PDC1*, *PDC2* and *ADH1* in pea roots untreated (control) or roots treated with pyruvate, imazamox, glyphosate or exposed to low-oxygen conditions grown in sterile conditions (3 days of treatment). Mean + SE (n=4). Significant variations are marked with ● for differences between control and imazamox-treated plants, with ● for differences between control and glyphosate-treated plants and with ◌ for differences between control and plants exposed to low-oxygen conditions (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).

As detected in the previous part of this chapter with other growing conditions, fermentation induction after ABIHs application was transcriptionally regulated. The relative transcript levels of *PDC1* increased after both herbicides while the mRNA level of *PDC2* and *ADH1* was only upregulated by GLP application. The low-oxygen stress also upregulated the transcription of the three tested genes.

Although the low-oxygen stress, IMX, GLP and pyruvate treatments induced the fermentation pathway, the pattern of induction and regulation was not equivalent, and significant differences were detected between the different treatments. Roots of pea plants grown in axenic boxes showed an increase in the transcription, in the protein amount and in the activity of the ethanol fermentation pathway when oxygen was removed for three days. A similar response was observed after both herbicide applications: an induction of the

transcription of both *PDC* and *ADH* genes, an increase in the amount of PDC and in the activity of ADH. The exogenous application of pyruvate resembled the effect of the herbicides with the important difference that no change in the transcript levels of *PDC1*, *PDC2* or *ADH1* were detected.

These results suggest that the induction of fermentation after IMX or GLP application was transcriptionally regulated. When pyruvate was applied, the increase in the activity of ADH, can be related to a higher flow of substrate into the ethanol pathway, due to an increase in the substrate availability for the PDC. It remains to be elucidated why a simultaneous increase of PDC activity was not detected together with the increase in the ADH activity. Moreover, the induction of the amount of PDC enzyme after pyruvate treatment despite no higher transcription level was detected, cannot be explained only by higher substrate availability and other post-transcriptional regulation mechanism have to be involved.

To evaluate whether pyruvate has the same transcriptional effects in another species, the transcript levels of *PDC1*, *PDC2* and *ADH1* were monitored in *A. thaliana* grown under sterile conditions. For that purpose, *A. thaliana* seedlings were grown in six-well petri dishes under sterile conditions and seedlings were treated with pyruvate, IMX or GLP for five days. Previous studies were conducted to determine that 5 days was the evaluation time point of the study. Specifically, the transcript levels of *ADH1* were monitored in *A. thaliana* seedlings treated with IMX or GLP at different time points and they were compared with the transcript levels of the control plants. The day at which the expression of *ADH1* was highest was selected as the time point of the study, which was the day 5 after herbicide application.

Figure 2.15 shows the aspect of the seedlings five days after the application of the different treatments. The supply of pyruvate promoted the growth of the seedlings and they were bigger than the untreated seedlings. By contrast, the growth of the seedlings was arrested as a consequence of herbicide application and the herbicide-treated seedlings were yellowish.

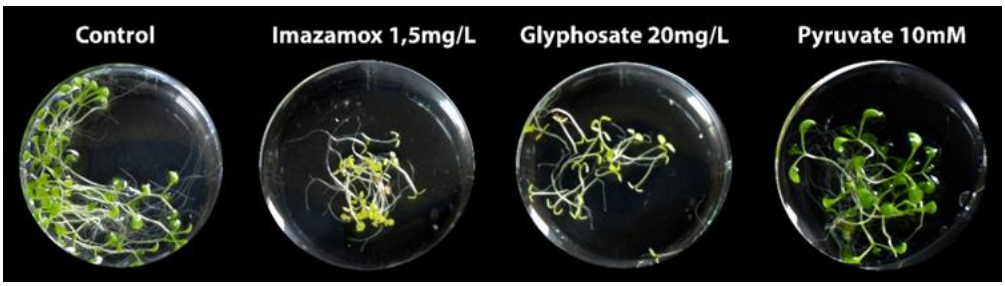


Figure 2.15. *Arabidopsis thaliana* seedlings untreated (control) or treated with pyruvate, imazamox or glyphosate for five days (grown in six-well plates under sterile conditions).

To evaluate whether the fermentative pathways upon herbicide or pyruvate treatment are transcriptionally regulated in *A. thaliana* seedlings grown under this growing system (after five days of treatment), the transcript levels of the genes *PDC1*, *PDC2* and *ADH1* were measured by qPCR (Figure 2.16). Moreover, the hypoxia marker *HB1* was included to check whether the exogenous supply of pyruvate in this growing system caused any effect in a typical marker of low oxygen conditions. No data regarding the *PDC2* transcript levels could be provided for the pyruvate treatment.

As it occurred in the roots of pea plants and in the roots of hydroponically grown *A. thaliana*, both herbicides induced the increases in *PDC1*, *PDC2* and *ADH1* mRNA. Interestingly, contrary to what it was observed in the roots of pea plants grown under sterile conditions, in the case of *A. thaliana* seedlings grown in axenic well plates, pyruvate also provoked an increase in the transcript levels of *PDC1* and *ADH1*. Nevertheless, the effect on *HB1* transcription was not parallel for herbicides and pyruvate, because while pyruvate did not change the transcription level of this hypoxia marker the application of ABIHs induced it.

The results shown in this part of the chapter show a complex regulation picture in the fermentation induction after pyruvate supply that cannot be simply explained as a mimicked effect of anaerobiosis. Pyruvate supply did not induce the expression of the typical hypoxia marker *HB1*, showing a different cascade from low-oxygen.

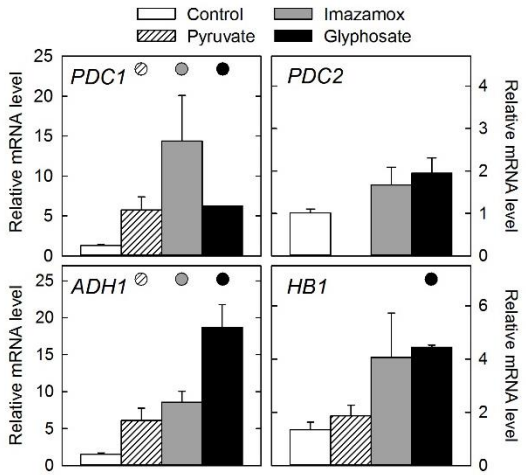


Figure 2.16. Relative transcript levels of the genes *PDC1*, *PDC2*, *ADH1* and *HB1* in *Arabidopsis thaliana* Col-0 seedlings grown in sterile conditions, untreated (control), or treated with pyruvate, imazamox or glyphosate for five days. Values represent the mean ± SE (n = 4 biological replicates). Significant variations are marked with ⊘ for differences between control and pyruvate-treated plants, with ◍ for differences between control and imazamox-treated plants, and with ● for differences between control and glyphosate-treated plants (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$). No data for the *PDC2* transcript levels of the pyruvate-treated seedlings could be provided.

The comparison of the effects of pyruvate and ABIHs on the fermentative activities was different depending on the used axenic experimental approach. While in pea roots pyruvate supply induced ADH activity and PDC protein content without an upregulation of the gene transcription, in *A. thaliana* seedlings, pyruvate induced the expression (both at transcript and protein levels) of *PDC1* and *ADH1*. This difference could be due to a different time point was used for the two experimental approaches (3 days for pea and 5 days for *A. thaliana*) and maybe, three days of pyruvate treatment were not enough to upregulate gene transcription. Besides, the lack of effects after pyruvate treatment in pea could be that the transcriptional regulation of the ethanol fermentation could be species-dependent. This was a conclusion of the Chapter 1 of this thesis, where the role of the induction of fermentation after herbicide treatment was different depending on the specie and the experimental approach.

2. 4. 3. Effect of herbicides on the N-end rule pathway mediated proteolysis and RAP2.12-regulated gene expression profile

Whether, as it occurs in low-oxygen stress, the TF RAP2.12 is involved in the transcriptional regulation of the ethanol fermentation in plants treated with IMX or GLP was evaluated. On the one hand, the possible effects of these herbicides on the NERP-mediated proteolysis was investigated in *A. thaliana* plants containing an artificial N-end substrate reporter (M-GUS or R-GUS reporter constructs). Moreover, the expression level of different genes (*HRA1*, *HUP7*, *PCO2* and *LBD41*) known to be upregulated by RAP2.12 upon low-oxygen stress, was monitored in *A. thaliana* Col-0 plants. To continue unravelling the complex role of pyruvate in the fermentative induction after ABIH treatment, the possible effects provoked by the exogenous supply of pyruvate on the NERP-mediated protein degradation and on the expression level of the RAP2.12-regulated genes were also evaluated.

To evaluate the effects of the herbicides and the exogenous supply of pyruvate on the NERP-mediated proteolysis, the stability of chimeric proteins expressed in *A. thaliana* seedlings containing artificial N-end substrates (R-GUS or M-GUS reporter constructs) was monitored by the GUS histochemical staining method (Figure 2.17). This assays is based on the ubiquitin fusion degradation technique, which was developed by Varshavsky (2005). This method allows the assessment of the stability of a test protein, since the plants developed by this method express a chimeric protein consisting of a ubiquitin moiety followed by a selected amino acid (X) attached to a reporter protein (in our case, GUS). The ubiquitin moiety present at the carboxyl end of the reference protein is recognized and cleaved by the deubiquitinating enzymes leaving the X amino acid exposed for degradation. The nature of this amino acid determines the stability of the protein, that it can be assessed detecting the reporter protein GUS. In our study, selected amino acids were Met (M) and Arg (R). Met comprises a stabilizing residue of the NERP in plants, thus, plants expressing the M-GUS chimeric protein will be stable under control conditions. By contrast, Arg is a destabilizing residue of the NERP in plants and thus the R-

GUS protein is degraded under control conditions while it has been shown to stabilize in *prt6* mutants (Garzón et al., 2007).

As expected, the M-GUS protein was stable in all the treatments and no degradation of this chimeric protein was observed (Figure 2.17). By contrast, the R-GUS protein was degraded in the control seedlings and its stability did not change after pyruvate or herbicide application and this protein was degraded upon all these treatments (contrary to what it would be expected in low-oxygen stress) (Figure 2.17). This results indicate that, opposite to what happens in low-oxygen-treated plants, the NERP-mediated proteolysis is not affected by these treatments and thereby, they potentially do not interfere with the degradation of RAP2.12.

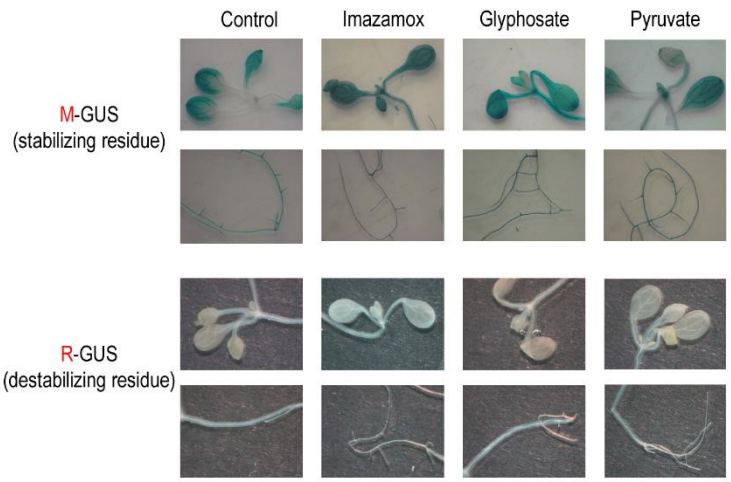


Figure 2.17. Effect of imazamox, glyphosate and pyruvate on the N-end rule pathway mediated proteolysis. Histochemical detection of GUS expression in *Arabidopsis thaliana* containing artificial N-end substrates reporters.

To confirm that the TF RAP2.12 is not involved in the transcriptional regulation of the ethanol fermentation in plants upon pyruvate or herbicide exposure, the expression of different RAP2.12-regulated genes were evaluated in *A. thaliana* seedlings treated with pyruvate, IMX or GLP for 5 days grown

under sterile conditions (Figure 2.18). No increase in the mRNA levels of the RAP2.12-regulated genes *(LBD41, PCO2, HRA1* and *HUP7)* was detected in the pyruvate- or herbicide-treated *A. thaliana* seedlings, except in the case of *PCO2* after IMX treatment. To validate the study, the transcript levels of these RAP2.12-regulated genes were monitored in the roots of *A. thaliana* plants exposed to low-oxygen conditions for three days (Figure 2.19). The results demonstrated that these genes were upregulated by low-oxygen conditions as it has been previously reported by other authors (Licausi et al., 2011a; Weits et al., 2014). These results confirm that the RAP2.12 is not involved in the transcriptional regulation of *PDC1*, *PDC2* and *ADH1* in plants upon herbicide or pyruvate treatment.

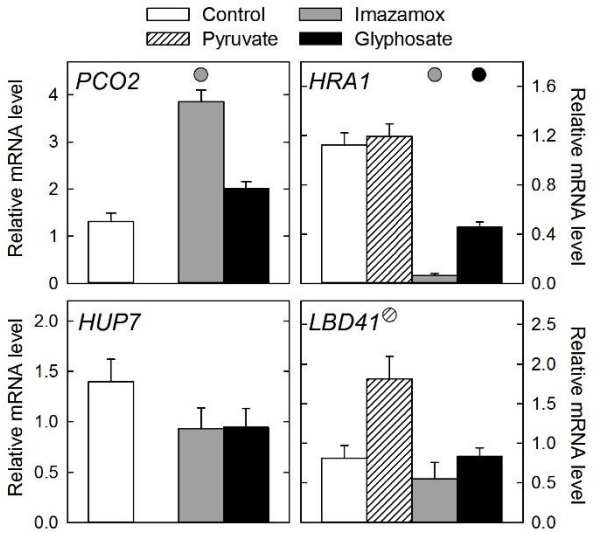


Figure 2.18. Relative transcript levels of the RAP2.12-regulated genes *PCO2*, *HRA1*, *HUP7 and LBD41* in pyruvate-, imazamox- or glyphosate-treated (for five days) *Arabidopsis thaliana* Col-0 seedlings grown under sterile conditions. Mean + SE (n=4). Significant variations are marked with ⊘ for differences between control and pyruvate-treated plants, with ● for differences between control and imazamox-treated plants, and with ● for differences between control and glyphosate-treated plants (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$). No data regarding the *PCO2* and *HUP7* transcript levels of the pyruvate-treated seedlings could be provided.

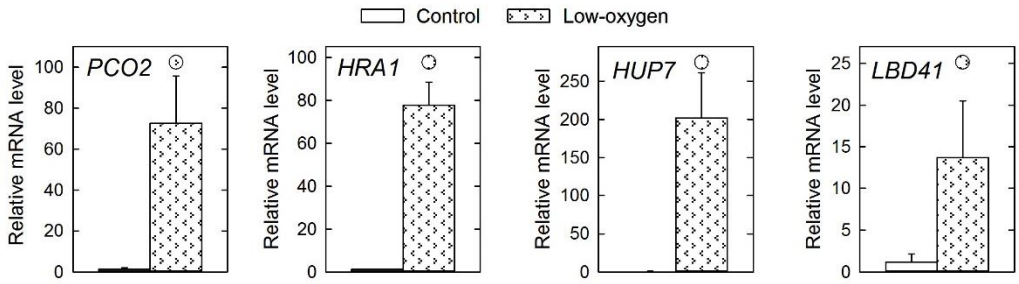


Figure 2.19. Relative transcript levels of different genes regulated by RAP2.12 (*PCO2*, *HRA1*, *HUP7* and *LBD41*) in the roots of *Arabidopsis thaliana* Col-0 plants untreated (control) or grown under low-oxygen conditions for three days. Mean + SE (n=4). Significant variations are marked with ⊙ (*t*-Test, $p < 0.05$).

It remains to be evaluated whether other transcription factors rather than RAP2.12 are implicated in the transcriptional regulation of the fermentative pathway upon treatment with herbicides. Studies conducted with different AHAS inhibitors reported an induction of the TF *HRE2* (Das et al., 2010), suggesting a possible role of this TF in the transcriptional regulation of *PDC1*, *PDC2* and *ADH1*. HRE2 has been shown to be able to induce the expression of the anaerobic genes under low-oxygen conditions and it has been associated to participate in the maintenance of the anaerobic response and not to be the initial activator (Licausi et al., 2010). While the anaerobic response is activated within 30 min upon hypoxia, fermentation takes longer to be induced after herbicide treatment. This could be indicating that, contrarily to what occurs in anaerobiosis, there is no need for a reservoir of the TF responsible for the activation of fermentation, and thus, other TF that are not constituvely expressed (as it is the case for HRE2) could be the ones triggering the induction of the fermentation pathways.

Additionally, the TF RAP2.3 could be another candidate for the transcriptional regulation of the ethanol fermentation in plants upon herbicide treatment, since it has been shown to play a role upon different stresses (Papdi et al., 2015). It would be interesting to study the role of RAP2.3 in the transcriptional regulation of fermentation upon treatment with herbicides

2. 5. CONCLUSIONS

In this chapter, the regulation of the ethanol fermentation in plants after ABIH treatment was evaluated, analysing the transcriptional regulation of the *PDC* and *ADH* genes, the possible role of pyruvate and the involvement of the RAP2.12 TF.

The main conclusions of this chapter are:

- The induction of enzymatic activities and protein level of the enzymes involved in ethanol fermentation detected in roots of pea and *A. thaliana* is transcriptionally regulated upon ABIH application, since the mRNA levels of the genes *PDC1*, *PDC2* and *ADH1* increased as a consequence of IMX or GLP application.
- The role of pyruvate in the induction of fermentation after ABIH treatment is a complex picture that cannot be simply explained by a mimicked effect or a higher substrate availability.
- The effect of pyruvate on the regulation of PDC and ADH induction was different depending on the plant species. In axenic pea plants pyruvate increased the PDC protein amount and ADH activity, while it did not change the transcript levels. On the contrary, in *A. thaliana* seedlings, the mRNA levels of *PDC1* and *ADH1* increased after pyruvate treatment.
- The accumulation of the PDC protein in pea roots after pyruvate treatment has to be due to an increase in the translation rate, because no increase in the relative transcript levels was detected.
- The TF RAP2.12 is not involved in the transcriptional regulation of the ethanol fermentation in plants after herbicide treatment or pyruvate supply. On the one hand, the NERP-mediated protein degradation was not affected by herbicides and pyruvate supply, thus, they likely do not interfere with RAP2.12 degradation. Moreover, no increase in the transcription levels of the RAP.2.12-regulated genes was observed by these treatments.

- CHAPTER 3 -

New insights in the common physiological response triggered by ABIHs: Aldehyde dehydrogenases and the PDH-bypass

3. 1. INTRODUCTION

3. 1. 1. Plant aldehyde dehydrogenases

ALDHs are a group of enzymes that catalyse the oxidation of aldehydes to their corresponding carboxylic acids in a reaction that consumes NAD(P^+) and produces NAD(P)H. Aldehydes are highly reactive molecules that are generated through normal cell metabolism but if they accumulate in excess they become toxic. Aldehydes are also generated in response to different stress conditions (such us, dehydration, salinity and cold stress) and under these circumstances, the activity of the ALDHs becomes crucial to avoid excess accumulation of aldehydes (Sunkar et al., 2003; Kotchoni et al., 2006).

In plants, the ALDH superfamily contains 14 distinct families (ALDH2, ALDH3, ALDH5, ALDH6, ALDH7, ALDH10, ALDH11, ALDH12, ALDH18, ALDH19, ALDH21, ALDH22, ALDH23 and ALDH24) from which seven (ALDH11, ALDH12, ALDH19, ALDH21, ALDH22, ALDH23 and ALDH24) are unique to plants (Zhang et al., 2012).

In *A. thaliana*, there are 16 genes encoding members of ten ALDH protein families (Stiti et al., 2011b; Brocker et al., 2013). The ALDH superfamily of *A. thaliana* is summarized in the Table 3.1.

While human ALDHs have been widely studied, plant ALDHs have been less explored and their precise physiological roles are often still unclear. The different ALDHs are known to participate in different cellular compartments and often in a tissue specific manner (Stiti et al., 2011b). The substrate specificity is also different for each protein, and while some ALDHs are substrate specific, others react with a variety of aldehydes (Brocker et al., 2013).

The interest in studying plant ALDHs is increasing since it has been shown that these proteins play essential roles in the plant growth, development, and the plant response to different stress conditions. Members of family 2, family 3 and family 7 have been described to play an important role in different plant stress responses (such as submergence, salinity and dehydration) (Nakazono et

al., 2000; Kirch et al., 2005; Kotchoni et al., 2006; Missihoun et al., 2012), and they will be described in detail in the following sections. Briefly, while members of the family 3 and family 7 ALDHs have been described to metabolize aldehydes derived from lipid peroxidation, members of family 2 ALDHs are known to contribute to the detoxification of the acetaldehyde produced during ethanol fermentation.

Protein family	Gene Name	Locus	Localization	Function
Family 2	***ALDH2B4***	**At3g48000**	Mitochondria	Pyruvate dehydrogenase bypass
	ALDH2B7	**At1g23800**	Mitochondria	
	ALDH2C4	**At3g24503**	Cytosol	Phenyl-propanoid pathway
Family 3	***ALDH3F1***	**At4g36250**	Cytosol	Stress-regulated detoxification pathway
	ALDH3H1	**At1g44170**	Cytosol	Stress-regulated detoxification pathway
	ALDH3I1	**At4g34240**	Chloroplasts	Stress-regulated detoxification pathway
Family 5	*ALDH5F1*	At1g79440	Mitochondria	Succinic semialdehyde dehydrogenase, involved in GABA-shunt pathway, stress-regulated detoxification of ROS intermediates, and in the patterning of *Arabidopsis* leaves along the adaxial–abaxial axis
Family 6	*ALDH6B2*	At2g14170	Mitochondria	Putative methylmalonyl semialdehyde dehydrogenase
Family 7	***ALDH7B4***	**At1g54100**	Cytosol	Turgor-responsive, stress-regulated detoxification pathway
Family 10	*ALDH10A8*	At1g74920	Leucoplasts	Putative stress-regulated AMADH, involved in the oxidation of aminoaldehydes derived from polyamine degradation
	ALDH10A9	At3g48170	Peroxisomes	
Family 11	*ALDH11A3*	At2g24270	Cytosol	Non-phosphorylating GAPDH
Family 12	*ALDH12A1*	At5g62530	Mitochondria	Δ^1-Pyrroline-5-carboxylate dehydrogenase, stress-regulated pathway essential for proline degradation, and protection from proline toxicity
Family 18	*ALDH18B1*	At2g39800	Mitochondria	
	ALDH18B2	At3g55610	?	
Family 22	*ALDH22A1*	At3g66658	Cytosol	Plant specific ALDH

Table 3.1. The ALDH superfamily in *Arabidopsis thaliana* (modified from Stiti et al., 2011b). In bold, the ALDHs studied in this work. ALDH, aldehyde dehydrogenase; AMADH, aminoaldehyde dehydrogenase; GABA, γ-aminobutyric acid; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ROS, reactive oxygen species.

a) Family 2 ALDHs

The ALDH2 gene family includes homotetrameric mitochondrial and cytosolic enzymes that react with a with range of substrates (Brocker et al., 2013). The first plant *ALDH2* gene identified encodes a nuclear restorer of cytoplasmic male sterility which corresponds with maize *ALDH2B2* (Cui et al., 1996).

The family 2 ALDHs have been proposed to metabolize the acetaldehyde generated as a consequence of ethanol fermentation under different circumstances. In rice, the expression of *ALDH2B5* has been shown to increase upon submergence, while no increase on the ALDH2B5 protein level has been detected under these circumstances (Nakazono et al., 2000; Tsuji et al., 2003; Sadiq et al., 2011). The detoxification of acetaldehyde to acetate by ALDH consumes NAD^+ and can potentially block glycolysis, fact that could explain why no increase in the ALDH2B5 protein level is detected during submergence (Nakazono et al., 2000). Nevertheless, the ALDH2B5 is a mitochondrial enzyme and probably, no competition for NAD^+ exists between the glycolysis and the ALDH activity (Nakazono et al., 2000). By contrast, ALDH2B5 protein has been described to accumulate during reaeration (Tsuji et al., 2003), suggesting that this protein may detoxify the acetaldehyde produced during reaeration by the oxidation of the ethanol produced during anoxia (Tsuji et al., 2003; Sadiq et al., 2011). The capacity to detoxify the acetaldehyde has been related to the submergence-tolerance, since the submergence-intolerant species (such as, maize) have been shown to be less able to detoxify acetaldehyde during reaeration (Meguro et al., 2006).

Role in the PDH-bypass

Members of tobacco family 2 ALDHs have also been proposed to detoxify the acetaldehyde produced during ethanol fermentation. An increase in the ethanol fermentation has been described during tobacco pollen development, which is primarily controlled by sugar supply rather than by oxygen availability

(Bucher et al., 1995; Tadege and Kuhlemeier, 1997). The inactivation of *ADH* in pollen did not affect normal pollen development, suggesting the existence of an alternative pathway to metabolize the acetaldehyde produced by PDC (Freeling and Bennett, 1985). Different studies support the presence of the PDH-bypass during pollen development (Mellema et al., 2002; Gass et al., 2005). This pathway is an alternative pathway to convert pyruvate into acetyl-CoA. While the main pathway consists of a single reaction catalysed by the PDH complex, the PDH-bypass involves the action of the enzymes PDC (conversion of pyruvate to acetaldehyde), ALDH (conversion of acetaldehyde to acetate) and acetyl-CoA synthetase (ACS) (conversion of acetate to acetyl-CoA) (Pronk et al., 1994) (Figure 3.1, shown in pink). The produced acetyl-CoA can be easily metabolized since it is the substrate for many different processes, such as, the TCA cycle in the mitochondria, the *de novo* fatty acid biosynthesis in the chloroplasts, the biosynthesis of secondary metabolites and elongation of fatty acids in the cytosol, and the glyoxylate cycle in the glyoxysomes (Figure 3.1). However, contrary to acetate, acetyl-CoA has a limited permeability through the membranes, and since no transporters for this metabolite have been found, it is likely that acetyl-CoA needs to be produced within each cellular compartment.

The existence of the PDH-bypass in tobacco pollen is supported by the fact that radiolabelled ethanol was shown to incorporate into CO_2, amino acids derived from intermediates of the TCA cycle and lipids (Mellema et al., 2002).

The PDH-bypass has also been shown to exist in vegetative tissues (Lin and Oliver, 2008; Wei et al., 2009). In *A. thaliana*, the mitochondrial ALDH2B4 was shown to participate in the PDH-bypass, since it was shown to participate in the incorporation of ^{14}C-ethanol into fatty acids (Wei et al., 2009). There are another two genes coding for family 2 ALDHs in *A. thaliana.* One of them, the *ALDH2C4*, codes for a cytosolic enzyme that plays an important role in the phenyl-propanoid pathway, being involved in ferulic acid and sinapic acid production during lignin biosynthesis (Nair et al., 2004). By contrast, the

ALDH2B7 encodes a mitochondrial enzyme whose physiological role has not been described yet (Stiti et al., 2011b).

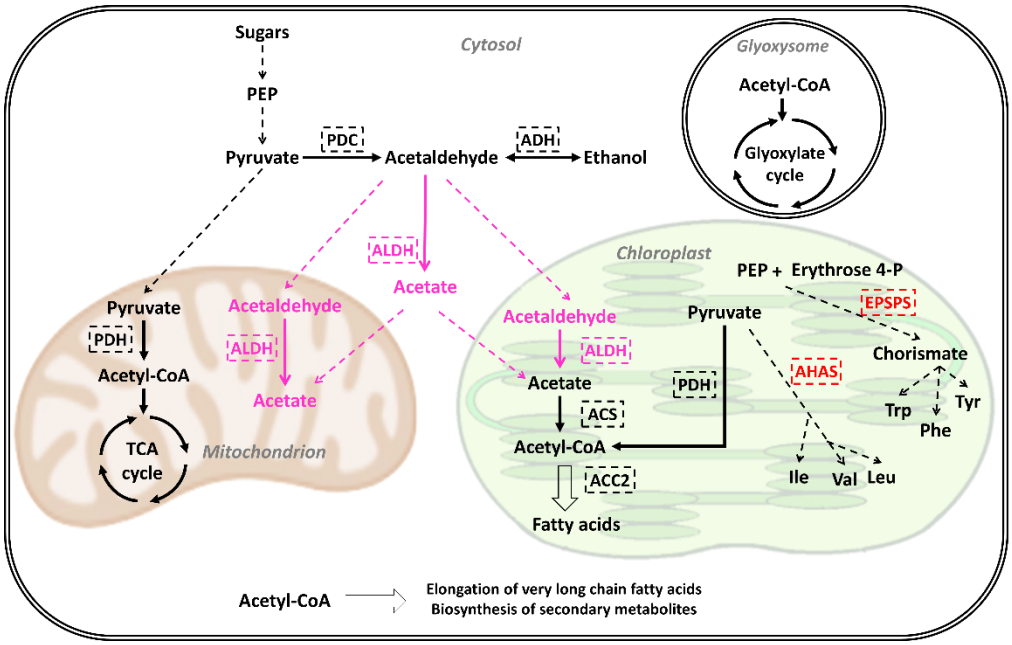


Figure 3.1. Overview of respiration, PDH-bypass (in pink), de novo fatty acid biosynthesis, branched-chain and aromatic amino acid biosynthesis, and acetyl-CoA-consuming pathways. ACC2, Acetyl-CoA carboxylase; ACS, acetyl-CoA synthetase; ADH, alcohol dehydrogenase; AHAS, acetohydroxyacid synthase; ALDH, aldehyde dehydrogenase, EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase; PDC, pyruvate decarboxylase; PDH, pyruvate dehydrogenase; PEP, phosphoenolpyruvate; TCA, tricarboxylic acid.

Apart from the role of the family 2 ALDHs in submergence and their participation in the PDH-bypass, it was shown that the expression of *ALDH2B4* isolated from Chinese wild grapevine (*Vitis pseudoreticulata*) in transgenic *A. thaliana* plants prevents mildew infection and increases salt stress tolerance (Wen et al., 2012).

b) Family 3 ALDHs

The family 3 ALDHs include dimeric enzymes located in the cytosol and microsomes and it is one of the most extensively distributed and diverse plant ALDH protein Families (Kirch et al., 2004; Brocker et al., 2013). The family 3

ALDHs have been widely studied in humans and they have been associated with carcinogenesis and genetic disorders (Yoshida et al., 1998).

There are three genes coding for ALDH3 proteins in *A. thaliana*, two of them (*ALDH3F1* and *ALDH3H1*) code for cytosolic enzymes, while the other one (*ALDH3I1*) encodes a chloroplastic enzyme. The substrate specificity was investigated in ALDH3H1 and ALDH3I1 and it was found that while the activity of ALDH3H1 only depends on NAD^+, ALDH3I1 is able to use both NAD^+ and $NADP^+$ as cofactors (Stiti et al., 2011a).

While the expression of the *ALDH3F1* does not change in response to any of the studied treatments (Kirch et al., 2005), both *ALDH3H1* and *ALDH3I1* are transcriptionally upregulated by different stress conditions (such as, dehydration, osmotic stress and exogenous ABA application) (Kirch et al., 2005; Kotchoni et al., 2006; Missihoun et al., 2012).

The stress-regulated expression of *ALDH3I1* is restricted to the leaves, while transcripts were almost undetectable in the roots (Kirch et al., 2005). Interestingly, the opposite stress-associated expression pattern is found for the *ALDH3H1* gene (Kirch et al., 2005).

Overexpression of the *ALDH3I1* gene improves stress tolerance, most likely by scavenging toxic aldehydes and thus reducing cellular lipid peroxidation (Sunkar et al., 2003; Kotchoni et al., 2006). By contrast, overexpression of the gene *ALDH3H1* did not improve stress tolerance, but the plants accumulated less malondialdehyde (MDA) derived from lipid peroxidation comparing to the wild-type plants (Missihoun et al., 2012).

Members of the family 3 ALDHs have been isolated in other plants, like the one isolated from *Craterostigma plantagineum* which was also found to be induced upon dehydration and ABA treatment (Kirch et al., 2001).

c) Family 7 ALDHs

The *ALDH7* gene family shows a high degree of conservation throughout evolution (Brocker et al., 2013). As it occurs for other plant *ALDHs*, the expression of *ALDH7* genes is upregulated by different stress conditions in several plant species, such as, *A. thaliana* (Kirch et al., 2005), soybean (Rodrigues et al., 2006), rice (Shin et al., 2009) and wheat (Chen et al., 2015).

There is only one gene coding for family 7 ALDHs present in *A. thaliana*, the *ALDH7B4* gene. This gene is strongly induced by exposition to different stresses (such as, dehydration, salinity, heavy metals and abscisic acid treatment) (Kirch et al., 2005; Kotchoni et al., 2006). This gene has been observed to contribute to stress tolerance since *A. thaliana* mutants overexpressing the *ALDH7B4* gene showed improved stress tolerance, fact that has been correlated with a decrease in the content of aldehydes derived from cellular lipid peroxidation (Kotchoni et al., 2006). Moreover, involvement of ALDH7B4 in stress tolerance was further corroborated in *A. thaliana ALDH7B4* T-DNA mutants that presented higher stress sensitivity (Kotchoni et al., 2006).

Ectopic expression of a soybean *ALDH7* gene in *A. thaliana* and tobacco plants increased abiotic stress tolerance in the transgenic plants and presented lower levels of MDA, confirming the protective role of ALDH7 proteins (Rodrigues et al., 2006). Additionally, rice plants lacking the *OsALDH7B6* gene, a gene necessary for seed maturation and maintenance of seed viability, showed higher stress sensitivity that have been related to an accumulation of MDA and of the yellow pigment oryzamutaic acid A (Shin et al., 2009). Moreover, *A. thaliana* plants ectopically overexpressing the wheat stress inducible *TraeALDH7B1-5A* showed improved tolerance to water deficit (Chen et al., 2015).

3. 1. 2. *De novo* fatty acid biosynthesis

In the plastids, the acetyl-CoA is the precursor for *de novo* fatty acid biosynthesis (Figure 3.2) (Li-Beisson et al., 2013). The acetyl-CoA, which is the building block for fatty acid production, is generated by the plastidial PDH complex. In a second reaction, the acetyl-CoA is metabolized to malonyl-CoA by the acetyl-CoA carboxylase 2 (ACC2). Before entering the fatty acid synthesis pathway, the malonyl group of malonyl-CoA has to be transferred from CoA to acyl carrier protein (ACP), in a reaction catalysed by a malonyl-CoA:ACP-malonyltransferase. Fatty acids are grown by sequential condensation of two-carbon units by enzymes of the fatty acid synthase complex. Acetyl-CoA is used as the starting unit and malonyl-ACP provides the two-carbon units at each step of elongation. For the synthesis of a C16 fatty acid, a four reaction (condensation, reduction, dehydration, and reduction)-containing cycle needs to be repeated seven times. The initial condensation reaction of acetyl-CoA and malonyl-ACP is catalysed by 3-ketoacyl-ACP synthase isoform III (KASIII), yielding a four-carbon product (3-ketobutyrl-ACP). For the next six turns of the cycle (up to 16:0-ACP), the condensation reaction is catalysed by isoform I of KAS. The produced 16:0-ACP can enter three possible reactions. First, it can be elongated to 18:0-ACP by an additional cycle of fatty acid synthesis catalysed by KAS II. Alternatively, the 16:0-ACP can enter the prokaryotic glycerolipid pathway or it can be hydrolysed by fatty acyl thioesterases to release free fatty acids that are exported from the plastid. Besides, most of the produced 18:0-ACP is desaturated by the stromal Δ^9 stearoyl-ACP desaturase. The resulting 18:1-ACP can either enter the prokaryotic glycerolipid pathway or be hydrolysed by fatty acyl thioesterases for export from the plastid.

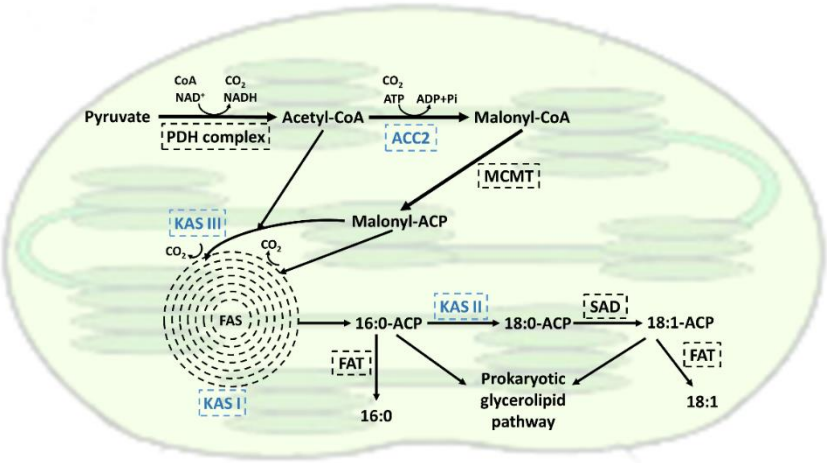


Figure 3.2. Overview of the *de novo* fatty acid biosynthesis (modified from Li-Beisson et al., 2013). In blue, the enzymes codified by the genes studied in the present work. ACC2, acetyl-CoA carboxylase; ACP, acyl carrier protein; FAS, fatty acid synthase; FAT, fatty acyl thioesterases; KAS, 3-ketoacyl-ACP synthases; MCMT, malonyl-CoA:ACP-malonyltransferase; PDH, pyruvate dehydrogenase; SAD, stromal Δ^9 stearoyl-ACP desaturase.

ABIHs and the PDH-bypass

Since the ethanol fermentation is induced in plants treated with ABIHs, an induction of the PDH-bypass could therefore be expected to collaborate in the detoxification of the acetaldehyde produced in the ethanol fermentation. And the acetyl-CoA derived from the PDH-bypass could be consumed in the *de novo* fatty acid biosynthesis. Herbicides have been described to affect fatty acid biosynthesis since a promotion of this pathway (via PDH complex) was reported in spinach chloroplasts treated with an AHAS inhibitor (Homeyer et al., 1985).

3. 2. OBJECTIVES

As it has been shown in the Chapter 1 of the present thesis, the induction of ethanol fermentation is one of the physiological responses triggered in plants as a consequence of ABIH application. Aerobic fermentation has been described to activate in pollen and in vegetative tissues in plants exposed to different stresses (Tadege et al., 1999). In addition, an induction of the PDH-bypass has been proposed as an alternative way to metabolize the acetaldehyde produced during ethanol fermentation (Mellema et al., 2002; Lin and Oliver, 2008). The fact that, the lack of the *ADH1* gene does not modify the response of the plants to herbicide application (as it has been observed in the Chapter 1 of the present thesis) opens the question for a possible existence of the PDH-bypass under these circumstances.

On the other hand, to our knowledge, no studies evaluating the role of ALDHs on the physiological effects triggered by ABIHs have been done. Different members of ALDHs are known to be important in the survival of the plants to different stresses, and they have been proposed to metabolize the aldehydes derived from lipid peroxidation.

The main objective of the Chapter 3 of the present thesis is **to evaluate whether selected ALDHs play a role in the response of the plants to AHAS and EPSPS inhibitors and to evaluate the presence of alternative pyruvate-consuming pathways.**

This general aim was approached by these specific objectives:

1. To test the importance of selected ALDHs in the response of the plants to AHAS or EPSPS inhibitors on ALDH families that have been related to stress situations, the expression pattern of the genes *ALDH2B4*, *ALDH2B7*, *ALDH2C4*, *ALDH3F1*, *ALDH3H1*, *ALDH3I1* and *ALDH7B4* was monitored in the leaves and the roots of *A. thaliana* plants after treatment with ABIHs.

2. To evaluate the role of *ALDH7B4* in the response of the plants to AHAS or EPSPS inhibitors, the more characteristic physiological effects provoked by ABIH application were evaluated in *A. thaliana* mutants lacking or overexpressing the gene *ALDH7B4*.

3. To check the possible existence of the PDH-bypass as a pyruvate-consuming pathway in plants after AHAS or EPSPS inhibition, the expression of different genes (*ACS*, *ACC2*, *KASI*, *KASII* and *KASIII*) involved in the fatty acid biosynthesis and the fatty acid content were monitored in *A. thaliana* wild-type, and mutants lacking or overexpressing the gene *ALDH7B4* and defectives in *ADH1* were used.

3. 3. MATERIALS AND METHODS

3. 3. 1. Plant material and herbicide treatments

a) *Hydroponic system*

A. thaliana Col-0 was used as the wild-type control. The *A. thaliana* Col-0 T-DNA mutants defective for *ADH1* (NASC ref. N552699, Banti *et al.*, 2008) or *ALDH7B4* (SALK line 143309, Alonso *et al.*, 2003; Kotchoni *et al.*, 2006) were kindly provided by Prof. van Dongen (Institute of Biology, RWTH Aachen University, Germany) and Prof. Bartels (IMBIO, University of Bonn, Germany), respectively. One transgenic line expressing *ALDH7B4* under the control of the CaMV 35S promoter and another one expressing the *ALDH7B4-promoter::GUS gene* cassette (Missihoun et al., 2014) were also donated by Prof. Bartels. The transgenic *A. thaliana* T-DNA mutant defective for *ADH1* used in the part II of the Chapter 1 of the present thesis was used in several comparisons. Seeds were surface sterilized and grown as described in section 1.3.1.b.1 of the Chapter 1. IMX or GLP treatments were applied to eight-weeks-old plants in the rosette stage (12-14 expanded leaves) as described in section 1.3.1.b.1 of Chapter 1.

Leaf and root samples were taken at day 3 after application of the treatments. Plant material was immediately frozen in liquid nitrogen and stored at -80°C for further analysis. Later, frozen samples were ground under liquid nitrogen using a Retsch mixer mill (MM200, Retsch®, Haan, Germany), the needed amount of tissue for each analysis was separated and stored at -80°C. Fresh material was used for the GUS staining.

b) *Axenic system*

Wild-type *A. thaliana* Col-0 and a double mutant of *A. thaliana* Col-0 with a T-DNA insertion line for *PDC1* and *PDC2* (*pdc1-pdc2*) (kindly provided by Francesco Licausi (PlantLab, Scuola Superiore Sant'Anna, Pisa, Italy) were used to obtain several results of this chapter. Seedlings were grown and treated under axenic conditions as described in the section 2.3.1.2.b of the Chapter 2 of the present thesis.

3. 3. 2. Semi-quantitative Reverse-Transcription-Polymerase Chain Reactions (RT-PCRs)

Total RNA from about 0.1 g of previously ground frozen leaf or root samples was extracted as described by Missihoun et al., 2012. Plant material was resuspended in 500 µL of the extraction buffer (6 M urea, 3 M LiCl, 0.01 M Tris-HCl (pH 8.0) and 20 mM EDTA (pH 8.0)) and 500 µL of phenol-chloroform-isoamyl alcohol (25:24:1) mixture was added to the homogenate. The mixture was centrifuged at 21,000 *g* for 5 min at 4°C and the upper aqueous phase was transferred to a new tube. Again, 500 µL of phenol-chloroform-isoamyl alcohol (25:24:1) mixture was added and the mixture was centrifuged at 21,000 *g* for 5 min at 4°C. The upper aqueous phase was transferred to a new tube and 500 µL of chloroform-isoamyl alcohol (24:1) mixture was added. The mixture was centrifuged as above and the upper phase was collected in a new tube. To precipitate the RNA, 50 µL of 3 M Na-acetate (pH 5.2) and 500 µL of cold isopropanol were added and, after carefully mixing, the tubes were kept on ice for 5 min. The mixture was centrifuged at 21,000 *g* for 10 min at 4°C and the supernatant was discarded. The pellet was washed twice with cold 70% ethanol. For each washing, 800 µL of 70% ethanol was added without dissolving the pellet and discarded after a centrifugation at 21,000 *g* for 5 min at 4°C. The pellet was air dried for a few minutes and resuspended in 25 µL RNase free water and stored at -80°C until further analysis.

Extracted RNA was subsequently quantified and analysed using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). OD 260 and 280 nm were read for every sample. The RNA quality was also checked in a 1% agarose gel. Five-fold diluted RNA samples were loaded onto a 1% agarose gel and run at 75 mA for 35 min. The gels were visualized in a Gel Doc™ 2000 system (Bio-Rad Laboratories Inc., Hercules, CA, USA).

For the PCR analysis 2 µg of total RNA was treated with 10 U RNase-free DNase I (Thermo Fisher Scientific Inc., Waltham, MA, USA) in 10 µL reaction

containing 1X DNase I buffer (10 mM Tris/HCl (pH 7.5), 0.5 mM $CaCl_2$ and 2.5 mM $MgCl_2$) at 37°C for 10 min. Then, 1 µL of 25 mM EDTA was added and the reaction was heated at 65°C for 10 min to deactivate the DNase I.

First-strand cDNA synthesis was performed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer's instructions.

After reverse transcription, RT-PCR was performed on an Eppendorf Mastercycler ep Gradient S (Eppendorf, AG, Hamburg, Germany). Each reaction was performed for 1 µL of cDNA in a total volume of 20 µL containing: 1x Ammonium Buffer (Tris-HCl (pH 8.5), $(NH_4)_2S0_4$, 15 mM $MgCl_2$, 1% Tween 20®) (Ampliqon A/S, Odense, Denmark), 0.2 mM of each dNTPs, 0.4 µM specific forward primer, 0.4 µM specific reverse primer and 1 U Taq polymerase (Ampliqon A/S, Odense, Denmark).

	FORWARD	REVERSE
ALDH2B4 (At3g48000)	cctcttcttcaaccaggggca	cttcgtctcgttcgccctct
ALDH2B7 (At1g23800)	aggtgacaggcttggttcca	ccaggcagggttcttgaggg
ALDH2C4 (At3g24503)	gggtgaaatttgcgtggcga	tgcgcatttgatcccttcct
ALDH3F1 (At4g36250)	gaagccatggaagctatgaaggagac	gtctctgtctctcactttccccctt
ALDH3H1 (At1g44170)	cgtttcgccggactatatcttgacg	tcaaccaactaagtccatgtttga
ALDH3I1 (At4g34240)	ctactggatgtgcctgaagcatc	catgagtctttagagaacccaaag
ALDH7B4 (At1g54100)	gaagcaatagccaaagacacacgc	gatatctcgattatcgtaggctcc
ACS (At5g36880)	gtcaaaggttcatggcccgg	tcgtctttggcaaccctggt
ACC2 (At5g16390)	cgtctctctgctaagcccaa	ctggagtaggtggggatggt
KASI (At5g46290)	tcctccaaacccacttcgct	aacccacggatctgaccacc
KASII (At1g74960)	cgtgatgggagagggagctg	tccgtatcgcctgcacagtt
KASIII (At1g62640)	agctccaatggctcggtgtt	atccccacgttaaaccggct
GUS	cgtcctgtagaaaccccaacc	gatagtctgccagttcagttcg
ACTIN2 (At3g18780)	ggaatccacgagacaacctataac	gaaacattttctgtgaacgattcct

Table 3.2. The list of primers used in the RT-PCRs.

The specific primers used for the RT-PCR analysis are detailed in Table 3.2. The parameters of the PCR programme were as follows: 5 min 94°C; 25-32 cycles

(30 s 94°C, 45 s 62°C and 2 min (3 min for *ALDH3F1*) 72°C); 5 min 72°C and pause at 4°C. The specific number of cycles used for each gene is presented in Table 3.3.

GENE	LEAVES	ROOTS
ALDH2B4	25 cycles	27 cycles
ALDH2B7	30 cycles	30 cycles
ALDH2C4	25 cycles	25 cycles
ALDH3F1	30 cycles	30 cycles
ALDH3H1	27 cycles	25 cycles
ALDH3I1	30 cycles	30 cycles
ALDH7B4	25 cycles	26 cycles
ACS	27 cycles	26 cycles
ACC2	27 cycles	28 cycles
KASIII	27 cycles	28 cycles
KASI	27 cycles	28 cycles
KASII	27 cycles	32 cycles

Table 3.3. Number of cycles used in the RT-PCRs for each specific primer pairs.

Two-fold diluted PCR amplified products were loaded in a 1% agarose gel and run at 135 mA for 35 min. A 1Kb Gene Ruler was used as control for the length of the bands. The gels were visualized in a Bio-Rad Gel Documentation Gel Doc™ 2000 System (Bio-Rad Laboratories Inc., Hercules, CA, USA) and to quantify the intensity of the bands given by the PCR amplified products the Quantity One® version 4.6.9 Software (Bio-Rad Laboratories Inc., Hercules, CA, USA) was used. The signal intensity value for each sample and for a specific gene was divided by that of the *ACTIN-2* gene for the same sample.

3. 3. 3. Polymerase Chain Reactions for the screening of the *aldh7b4* mutants

Genomic DNA was extracted from about 0.1 g of previously frozen leaves. The plant material was homogenized in 375 µL of 2× lysis buffer (0.6 M NaCl, 0.1 M Tris-HCl (pH 8.0), 40 mM EDTA (pH 8.0), 4% sarcosyl, and 1% SDS) and 375 µL of 2 M urea. One volume (750 µL) of phenol/chloroform/isoamyl alcohol (25:24:1) was added to the mixture and mixed briefly. The homogenates were centrifuged at 20,000 g for 10 min at room temperature. To precipitate the DNA,

0.7 volume (525 µL) of cold isopropanol was added to the supernatants, and the tubes were centrifuged at 20,000 g for 15 min at 4 °C. The DNA pellet was washed twice with 1 mL of 70% ethanol, air-dried, and resuspended in 25 µL of resuspension buffer (10 mM Tris-HCl (pH 8.0), containing 30 µg mL–1 RNase A). Samples were briefly incubated at 37 °C for 5 min to degrade contaminating RNAs.

Extracted DNA was subsequently quantified and analysed using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). OD 260 and 280 nm were read for every sample. The DNA quality was also checked in a 1% agarose gel. Ten-fold diluted DNA samples were loaded onto a 1% agarose gel and run at 75 mA for 35 min. The gels were visualized in a Gel Doc™ 2000 system (Bio-Rad Laboratories Inc., Hercules, CA, USA).

A PCR was performed on an Eppendorf Mastercycler ep Gradient S (Eppendorf, AG, Hamburg, Germany). Each reaction was performed for 2 µL of genomic DNA in a total volume of 10 µL containing: 1x PCR Buffer (Takara Bio Inc., Shiga, Japan), 2.5 mM $MgCl_2$, 0.25 mM of each dNTPs, 0.6 µM specific forward primer, 0.25 µM specific reverse primer and 0.25 U Takara Taq polymerase (Takara Bio Inc., Shiga, Japan).

The specific primers used are detailed in Table 3.4. The parameters of the PCR carried out for the screening of the *aldh7b4* mutants were as follows: 5 min 95°C; 34 cycles (30 s 95°C and 8 min 30 s 68°C); 10 min 68°C and pause at 4°C.

Screening of *aldh7b4* mutants

	FORWARD	REVERSE
ALDH7B4 (At1g54100)	catacgaggatgatcgtggcaatgt	
T-DNA		cagtcatagccgaatagcctctcca

Table 3.4. The list of primers used in the PCRs.

Five-fold diluted PCR amplified products were loaded in a 1% agarose gel and run at 135 mA for 35 min. A 1Kb Gene Ruler was used as control for the

length of the bands. The gels were visualized in a Bio-Rad Gel Documentation Gel Doc™ 2000 System (Bio-Rad Laboratories Inc., Hercules, CA, USA).

3. 3. 4. Histochemical detection and measurement of the GUS activity

GUS staining was performed as described by Jefferson et al. (1987) with minor modifications. Fresh leaves or roots were incubated overnight at 37°C in X-Gluc solution (0.05% (w/v) X-Gluc (previously dissolved in N'N-Dimethyl-Formamide), 0.1% (v/v) Triton X-100 and 50 mM $NaPO_4$ (pH 7.0)). Then, the leaves were destained in EtOH 80%, by incubating them at 80°C for 20-30 min to remove the chlorophyll from leaves. The samples were observed and scanned in a GT-15,000 EPSON scanner (Seiko Epson Corporation, Suwa, NGN, Japan).

Fluorometric GUS activity of crude plant extracts was determined according to Jefferson et al. (1987) with minor modifications. About 0.1 g of previously ground shoots or roots was suspended in 200 µL of extraction buffer (50 mM sodium phosphate (pH 7.0), 10 mM EDTA, 0.1% (v/v) TritonX-100, 0.1% (w/v) Na-lauryl sarcosine and 10 mM β-mercaptoethanol). Samples were subsequently centrifuged at 21,000 *g* for 5 min at 4°C and the supernatant was transferred to a new tube. The protein concentration of the crude extract was determined from 5 µL of each sample by the Bradford assay (Bradford, 1976).

Then, 15 µL (*sample volume*) plant extract was mixed with 15 µL 1 mM 4-MUG and the reaction was incubated at 37°C. A control reaction was made without plant extract. Five microlitres (*volume per test*) were removed periodically from each reaction and diluted in 2 mL (r*eaction volume*) stop buffer consisting of 0.2 M Na_2CO_3 (pH 9.5). The fluorescence intensity (FI) of the samples was read in a fluorometer (Bio-Rad Laboratories Inc., Hercules, CA, USA) using 365 nm excitation and emission at 455 nm filters. A standard curve containing known concentrations of 4-MU ranging from 5-100 nM was generated to extrapolate the 4-MU values of the samples according to their FI values. The slope x (FI/pmol 4-MU) was calculated. Also, the slope y (FI/min) generated after plotting the FI values of the samples versus time was calculated. Each y-value was corrected by subtracting the y-value of the control reaction.

The specific GUS activity for each sample was expressed as 4-MU pmol/min/mg protein.

3. 3. 5. Lipid peroxidation assay

The level of lipid peroxidation products was measured in the plant tissues by the thiobarbituric acid (TBA) test according to Hodges et al. (1999). The previously ground plant tissues (leaves or roots) (50-60 mg) were homogenized in 1 mL pre-chilled 0.1% (w/v) trichloroacetic acid solution. The homogenates were centrifuged at 20,000 *g* for 5 min at 4°C. Then, 750 µL of the supernatant were mixed with 750 µL of the Reagent Solution II (RSII: RSI (20% w/v trichloroacetic acid and 0.01% Butylated hydroxytoluene (BHT)) + 0.65% TBA), and after vigorously mixing the tubes, they were boiled at 95°C for 25 min in a water bath. The reaction was stopped by placing the tubes on ice. The samples were centrifuged as above and supernatants were transferred to new tubes. Absorbances were read with a Sinergy™ HT Multi-Detection Microplate Reader (BioTek Instruments Inc., Winooski, VT, USA) at 440 nm (sugar absorbance), 532 nm (maximum absorbance of pinkish-red chromagen, product of the reaction of MDA with TBA) and 600 nm (turbidity). A reference solution was used consisting of 0.1% (w/v) trichloroacetic acid. The MDA contents were estimated by the following formula:

MDA equivalents (nmol mL^{-1}) = [(A–B)/157 000] x 10^6 where A = [(Abs 532_{RSII} – Abs 600_{RSII})] and B = [(Abs 440 $_{RSII}$ – Abs 600 $_{RSII}$) x 0.0571].

MDA equivalents (nmol g^{-1} FW) *= MDA equivalents (nmol mL^{-1}) x total volume of the extracts (mL) /* g *FW.*

3. 3. 6. Glutathione content

Glutathione content was determined from frozen leaves and roots as described in section 1.3.8 of Chapter 1.

3. 3. 7. PDC and ADH activities

The *in vitro* activities of PDC and ADH were assayed in ground leaf and root samples as described in section 1.3.3 of Chapter 1.

3. 3. 8. Free Amino Acid Extraction and Determination

Total free amino acids were measured from ground leaf or root samples as described in section 1.3.7.b of the Chapter 1 of the present thesis.

3. 3. 9. Carbohydrate Extraction and Determination

Ethanol-soluble sugars (glucose, sucrose and fructose) and starch content were determined as described in section 1.3.6 of the Chapter 1 of the present thesis.

3. 3. 10. Organic Acid Extraction and Determination

Long chain and short chain organic acid were measured from ground leaves and roots as described in section 1.3.9 of Chapter 1.

3. 3. 11. Quantitative Real-Time-Polymerase Chain Reactions

qPCRs were performed in axenically grown wild-type *A. thaliana* plants and in the *pdc1-pdc2* mutants as described in section 2.3.4 of the Chapter 2 of the present thesis. The specific primers used are presented in Table 3.5.

qPCR

	FORWARD	REVERSE
ALDH7B4 (At1g54100)	gagccgacaactcaatggatcg	tgccaagaggattccacatctcc
ACS (At5g36880)	aagagatgtgtggtggcaggatg	ccattccacctcacacgatgttgg
ACTIN2 (At3g18780)	tcttccgctctttctttccaagc	accattgtcacacacgattggttg

Table 3.5. The list of primers used in the qPCRs.

3. 3. 12. Total Fatty Acid Extraction and Determination

For Total fatty acid extraction glass tubes with screw caps containing teflon septa were used. About 0.1 g FW of ground leaf or root sample was immediately submerged in 1 mL of 1 M HCl in methanol containing 5 µg of pentadecanoic acid (15:0), used as the internal standard. The homogenates were incubated at 80°C for 20 min in an oven. Once the samples were again at room temperature, 1 mL of 0.9% NaCl and 1 mL of hexane were added. The tubes were centrifuged at 3,000 *g* for 3 min at room temperature, and the upper phase was transferred to the sample tube used for the gas chromatography. Samples were concentrated under a N_2 stream.

The fatty acid methyl esters were quantified by gas chromatography with flame ionization detector on an Agilent 7890A Gas Chromatograph (Agilent Technologies Inc., Santa Clara, CA, USA).

3. 3. 13. Statistical analysis

The data obtained from this study were analysed by the IBM SPSS Statistics (v.22). The mean was used as a measure of central tendency and the SE as a measure of dispersion.

First, for each studied parameter, the untreated plants of each genotype were compared to the untreated wild-type plants by the Student's *t*-test for the Significance of the difference between the means of two independent samples, at a significance level of 5% ($p < 0.05$).

Second, the data of the herbicide-treated and non-treated plants of each genotype were compared using the one-way ANOVA, after log transformations of the data if needed. In order to confirm homoscedasticity of variances, the Levene's test was used. The HSD Tukey and Dunnett T3 *post hoc* statistical tests were applied to the homogeneity and non-homogeneity of variances cases, respectively. When the results were expressed in percentages, the data were previously transformed according to the following formula: $arcsin\sqrt{x/100}$. In all cases, statistical analyses were conducted at a significance level of 5% ($p < 0.05$).

3. 4. RESULTS AND DISCUSSION

3. 4. 1. Profile of ABIHS on the expression level of selected *ALDH* genes

ALDHs are known to contribute to different stress tolerance since they detoxify aldehydes produced in plants exposed to several stress conditions. Among the different ALDH families existing in plants, members belonging to family 2, family 3 and family 7 have been described to play a role in the response of the plants to different abiotic stresses (such as flooding, salinity and dehydration) (Nakazono et al., 2000; Kirch et al., 2005; Rodrigues et al., 2006).

To our knowledge, no study evaluating the role of ALDHs in the response of the plants to herbicide exposure has been performed until now. Thus, little information is available regarding the effect of herbicides on the plant ALDHs. A proteomic approach revealed a decrease in the content of a rice family 2 mitochondrial ALDH after exposition to GLP (Ahsan et al., 2008).

To investigate whether ABIH application has an effect on the plant ALDHs, the transcription levels of the *ALDH* genes belonging to family 2 *(ALDH2B4, ALDH2B7* and *ALDH2C4*), family 3 (*ALDH3H1, ALDH3I1*) and family 7 (*ALDH3F1* and *ALDH7B4*) were analysed by RT-PCRs in the leaves and roots of IMX- or GLP- treated wild-type *A. thaliana* Col-0 plants (Figure 3.3 and Figure 3.4).

The results demonstrated that the upregulation of *ALDH7B4* was the only common effect triggered by both herbicide treatments. The expression of *ALDH7B4* was induced by IMX or GLP application in both studied organs, with GLP having stronger induction than IMX. *ALDH7B4* has been described to play a role under different stress conditions in several plant species (such as, rice, wheat, *A. thaliana* and soybean) (Kirch et al., 2005; Rodrigues et al., 2006; Shin et al., 2009; Chen et al., 2015). The overexpression of this gene was found to increase stress tolerance, probably by detoxifying aldehydes derived from lipid peroxidation (Kotchoni et al., 2006; Rodrigues et al., 2006).

On the other hand, the expression of the *ALDH2C4* increased in the leaves of IMX-treated plants. This gene encodes a protein known to play a role in the biosynthesis of ferulic acid and sinapic acid (Nair et al., 2004). This effect suggests a higher synthesis of these compounds, which indeed have been reported to accumulate in imidazolinone-treated pea plants (Orcaray et al., 2011).

As it has been introduced in the discussion of the Chapter1 of the present thesis, ethanol fermentation is induced in plants treated with IMX or GLP, so an induction of the PDH-bypass could be expected in plants upon ABIH-treatment. *A. thaliana* family 2 ALDHs were examined for their participation in the PDH-bypass and *ALDH2B4* was observed to be the primary contributor to this pathway in both vegetative and floral tissues (Wei et al., 2009). However, our results showed that the expression of *ALDH2B4* is not induced as a consequence of ABIH application (Figure 3.3 and Figure 3.4).

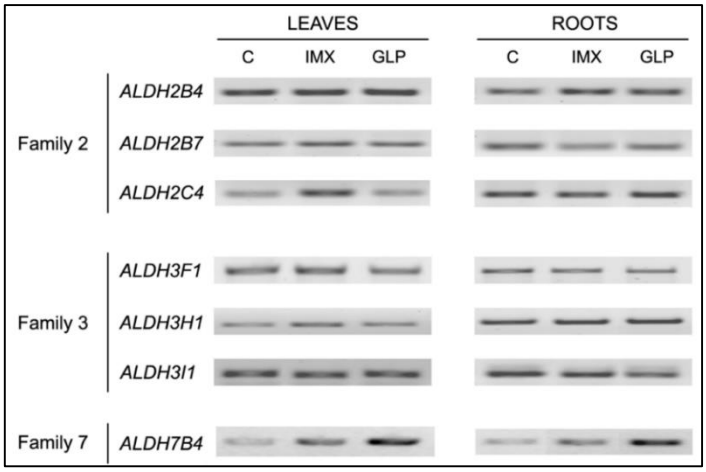


Figure 3.3. Expression patterns of the different *ALDH* transcripts under herbicide treatments. Relative transcript levels of the *ALDH2B4, ALDH2B7, ALDH2C4, ALDH3F1, ALDH3H1, ALDH3I1* and *ALDH7B4* genes in the leaves and the roots untreated (C) or in IMX- or GLP-treated wild-type *Arabidopsis thaliana* Col-0 plants (3 days after application). Gels shown are representative examples of the six RT-PCR assays. C, control; GLP, glyphosate; IMX, imazamox.

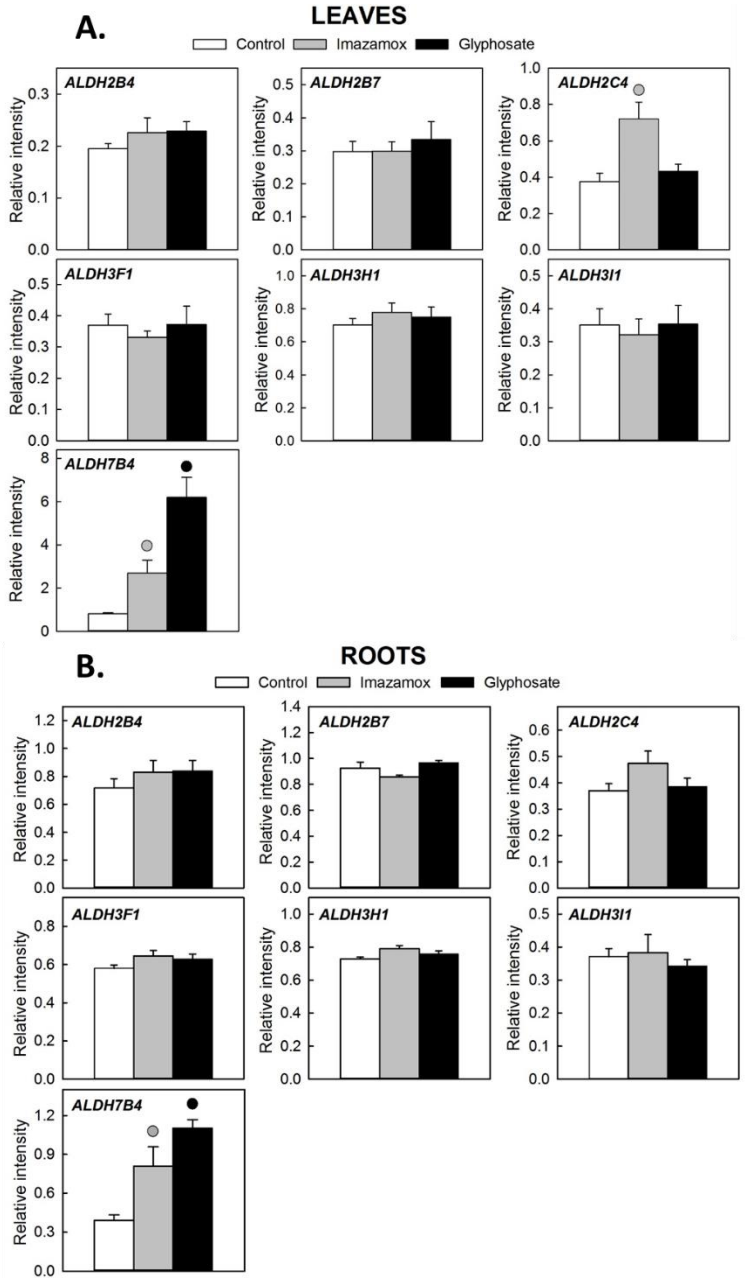


Figure 3.4. Relative transcript levels of the *ALDH2B4, ALDH2B7, ALDH2C4, ALDH3F1, ALDH3H1, ALDH3I1* and *ALDH7B4* genes in the leaves (A) and the roots (B) of imazamox- or glyphosate-treated wild-type *Arabidopsis thaliana* Col-0 plants (3 days after application). The relative band intensity from Figure 3.3 was measured with the Quantity One software (Bio-Rad Laboratories Inc., Hercules, CA, USA).Values represent the mean ± SE (n = 6). Significant variations are marked with ○ for differences between control and imazamox-treated plants, and with ● for differences between control and glyphosate-treated plants (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).

3. 4. 2. Confirmation of ALDH7B4 promoter activity confirmed that the expression of this gene increases upon ABIH application

To confirm the induction of *ALDH7B4* after herbicide treatment, *ALDH7B4-promoter::GUS* transgenic plants were grown and treated with IMX or GLP and the promoter activity was monitored by histochemical detection and measurement of the GUS enzyme activity (Figure 3.5).

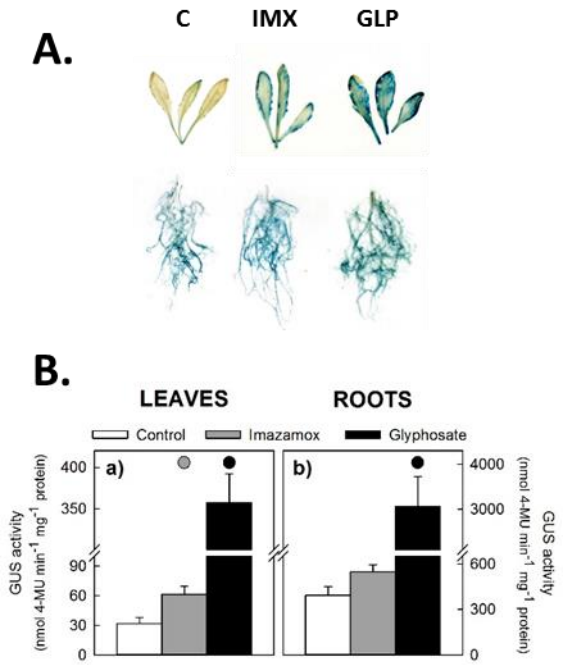


Figure 3.5. Activity of the *ALDH7B4* promoter upon herbicide treatments. **A.** *In situ* detection of the activity of *ALDH7B4* promoter in leaves and roots of herbicide-treated *A. thaliana* plants. **B.** Measurement of the *ALDH7B4* promoter-driven GUS activity in leaves and roots of *ALDH7B4::GUS Arabidopsis thaliana* plants treated with IMX or GLP. Values represent the mean ± SE (n = 6). Significant variations are marked with ○ for differences between control and imazamox-treated plants, and with ● for differences between control and glyphosate-treated plants (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$). C, control; GLP, glyphosate; IMX, imazamox.

It was found that the activity of the promoter of *ALDH7B4* is strongly induced in both leaves and roots of IMX or GLP treated plants (Figure 3.5). The activity of the *ALDH7B4* promoter dramatically increased after GLP treatment while the increase detected after IMX application was milder. Changes in the *GUS* expression in the roots of *ALDH7B4::GUS* transgenic plants were not

obvious by the histochemical GUS staining method. This was probably due to the high GUS activity already present in the roots of untreated plants.

RT-PCRs were also conducted for the *ALDH7B4* and *GUS* genes in the leaves and the roots of *ALDH7B4::GUS* transgenic plants (Figure 3.6). The *GUS* and *ALDH7B4* transcript levels of both leaves and roots increased as a consequence of both herbicide treatments. GLP was the herbicide that caused a significant increase in the expression of the two genes.

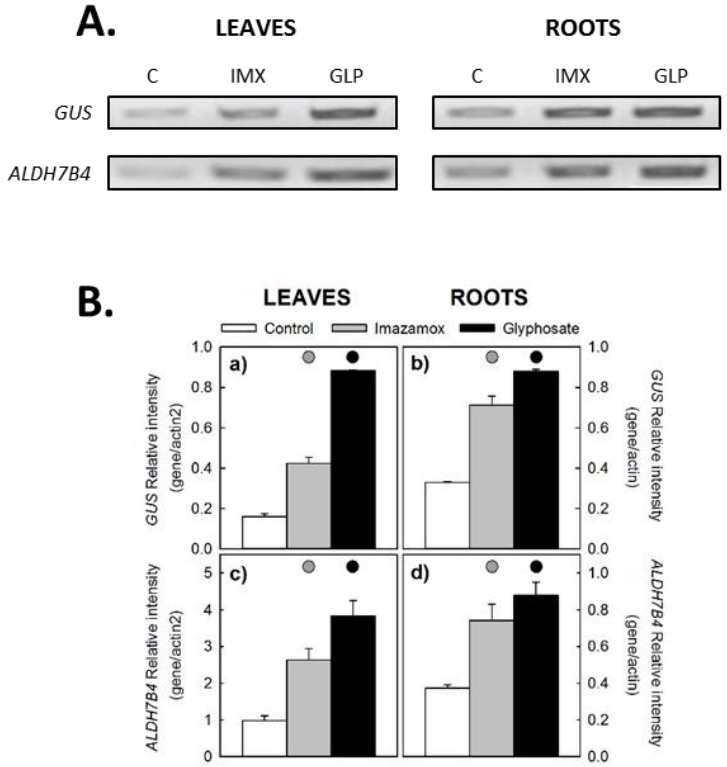


Figure 3.6. Expression patterns of the *GUS* and *ALDH7B4* transcripts under herbicide treatment. Relative transcript levels of the *ALDH7B4* and *GUS* genes in leaves and roots of untreated or IMX- or GLP- treated *ALDH7B4::GUS Arabidopsis thaliana* plants (3 days after application). The relative band intensity was measured the using Quantity One software (Bio-Rad Laboratories Inc., Hercules, CA, USA). Values represent mean ± SE (n=6). ● and ● indicate significant difference between C and IMX- or GLP-treated plants, respectively (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$). Gels shown are representative examples of the six RT-PCRs carried out. C, control; GLP, glyphosate; IMX, imazamox.

The results observed in the analysis of the *ALDH7B4* promoter correlate with the ones obtained in the *ALDH7B4* gene expression analysis carried out in wild-type *A. thaliana* Col-0 plants. The high induction of the expression of the *ALDH7B4* indicates that it plays an important role in plants upon ABIH-treatment.

3. 4. 3. Role of ALDH7B4 in the physiological response after ABIH treatment

The induction of the expression of *ALDH7B4* and the increase in the activity of its promoter suggest that this ALDH is important in the response of the plants to ABIH application. Interestingly, it was common to both types of herbicides supporting the hypothesis of this thesis, that herbicides inhibiting two different pathways of amino acid biosynthesis (branched chain and aromatic) provoke plant death by similar mechanisms.

To evaluate the importance of *ALDH7B4* in the response of the plants to IMX or GLP application, a similar experimental approach to the one presented in the Part II of the Chapter 1 of the present thesis was performed. The effects of the herbicides on different parameters were compared between wild-type and two transgenic lines: *A. thaliana* T-DNA mutants defective for *ALDH7B4* (*aldh7b4*) and a transgenic line expressing *ALDH7B4* under the control of the CaMV 35S promoter (*35S::ALDH7B4*).

First of all, both mutant lines were validated. On the one hand, the presence of the T-DNA in the *aldh7b4* mutants was confirmed. A PCR was performed in the genomic DNA using an *ALDH7B4* specific primer plus a T-DNA specific primer (Figure 3.6); the wild-type plants were used as negative control. The results confirmed the presence of the T-DNA insertion in the *aldh7b4* mutants, while no presence of the T-DNA was detected in the wild-type plants (Figure 3.7).

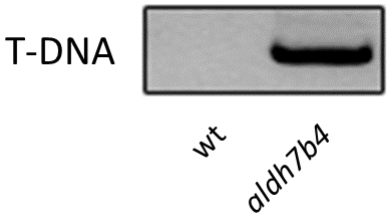


Figure 3.7. Screening of the T-DNA insertion in *Arabidopsis thaliana* wild-type (wt) and the T-DNA insertion mutants defective for *ALDH7B4* (*aldh7b4).*

On the other hand, the expression level (mRNA transcript level) of the *ALDH7B4* gene was also monitored in the three studied genotypes by RT-PCR (Figure 3.8). The results demonstrated that the *ALDH7B4* overexpressing line (*35S::ALDH7B4*) presented higher transcript levels of the *ALDH7B4* than the wild-type plants in both leaves and roots. Besides, no expression of the *ALDH7B4* was detected in the *aldh7b4* mutants. Collectively, these results validated the *aldh7b4* and the *35S::ALDH7B4* mutant lines.

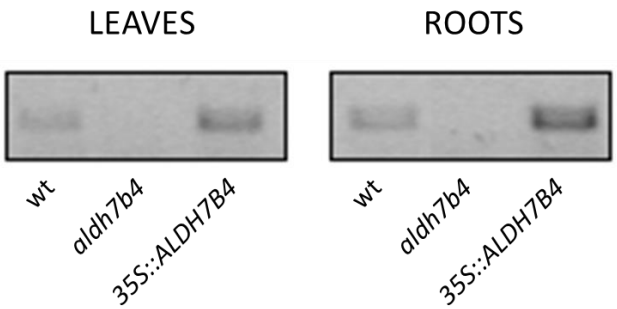


Figure 3. 8. Comparative analysis of the accumulation of the *ALDH7B4* transcripts in the leaves and the roots of wild-type (wt) *Arabidopsis thaliana*, the T-DNA insertion mutants defective for *ALDH7B4* (*aldh7b4)* and the *ALDH7B4* overexpressor line (*35S::ALDH7B4*).

To evaluate the role of the ALDH7B4 in the response of the plants to ABIH application, three potential functions were examined regarding the effects of ABIHs on the mutants:

- *Role in alleviating a possible oxidative stress induced by the herbicides.* This hypothesis is based on the fact that *ALDH7B4* gene was found to increase stress tolerance, probably by detoxifying aldehydes derived from lipid peroxidation (Kotchoni et al., 2006; Rodrigues et al., 2006).
- *Attenuation or enhancement of the toxicity of the herbicides*. The more characteristic physiological effects provoked by ABIH application (induction of ethanol fermentation, carbohydrate and amino acid accumulation, increase/decrease in different organic acid contents) were monitored in the leaves and the roots of *A. thaliana aldh7b4* and *35S::ALDH7B4* mutants and they were compared with the effects provoked in wild-type plants. This way, it could be monitored whether the lack or the overexpression of the *ALDH7B4* gene attenuates or enhances the response after ABIHs application.
- *Role in a possible PDH-bypass involved in the fatty acid synthesis.* Acetaldehyde produced by the PDC induction after ABIH treatment can be proposed to be further metabolized to acetyl-CoA by the PDH-bypass and support lipid biosynthesis as has been proposed in other tissues under aerobic conditions in tobacco pollen (Gass et al., 2005). Acetyl-CoA can follow different routes in the different cells compartments. In the plastids, acetyl-CoA is a substrate for the *de novo* fatty acid biosynthesis (Figure 3.2).

No phenotypical differences were found between the defective and overexpressing lines used in the analysis of the role of *ALDH7B4*. First of all, for each parameter a comparison between the untreated plants from each mutant line and the untreated wild-type plants was done. For almost all the parameters used in the evaluation of the three potential roles, no differences between the untreated plants of each genotypes were found.

a) *ALDH7B4* is not related to an alleviation of an oxidative stress

To evaluate whether ALDH7B4 detoxifies the aldehydes derived from lipid peroxidation upon ABIH treatment, the MDA content was measured in the leaves and the roots of untreated and herbicide-treated wild-type *A. thaliana* plants and they were compared to the contents in the *aldh7b4* and the *35S:ALDH7B4* mutants (Figure 3.9).

The results showed that the MDA content only increased in the leaves of GLP-treated wild-type plants, and no increase in the MDA content was found in the roots of IMX or GLP treated plants. These results reveal that lipid peroxidation only increases in leaves as a consequence of GLP application and that, by contrast, IMX application does not provoke lipid peroxidation. The transgenic lines showed the same pattern found in the wild-type plants, indicating that the MDA content is not influenced by the lack or the overexpression of the *ALDH7B4* gene in ABIH-treated plants.

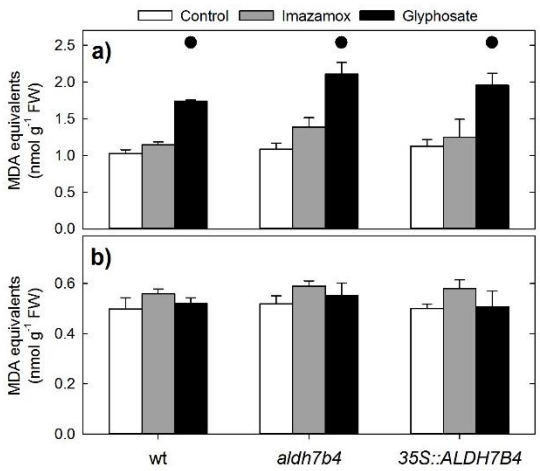


Figure 3.9. As a marker of lipid peroxidation, the malondialdehyde (MDA) content was measured in the leaves (a) and the roots (b) of wild-type (wt) *Arabidopsis thaliana* Col-0, *aldh7b4 and 35S::ALDH7B4* mutant plants, untreated (control) or treated with imazamox or glyphosate for 3 days. Values represent the mean ± SE (n = 5 biological replicates). Significant variations are marked with ○ for differences between control and imazamox-treated plants, and with ● for differences between control and glyphosate-treated plants (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).

Similar effects of ABIHs on oxidative indicators have been reported before. Studies evaluating different oxidative markers and antioxidant systems in plants treated with imazethapyr (another imidazolinone), demonstrated that the application of this type of herbicides do not provoke an oxidative stress (Zabalza et al., 2007). By contrast, GLP application has been previously related with an oxidative stress in plants (Ahsan et al., 2008; Miteva et al., 2010), and our results correlate with these previous studies, since the MDA content increased in leaves GLP-treated plants. Nevertheless, the role of *ALDH7B4* seems not to be related to the detoxification of aldehydes derived from lipid peroxidation because lipid peroxidation was not a common effect of both types of herbicides and no differences were detected when comparing the different mutant lines.

The glutathione content was also measured as an indication of the oxidation level in the cells (Figure 3.10). The glutathione is an antioxidant that protects the cells from reactive oxidative damage, since it has been described to participate in the redox-homeostatic buffering (Noctor et al., 2011) and this compound has been shown to be important in herbicide detoxification (Dixon and Edwards, 2010). It was evaluated the effects of the herbicides on the content of GSH, GSSG, total glutathione and the ratio between GSH/GSSG (Figure 3.10).

The results showed that both herbicides provoked an increase of the total glutathione and GSH contents in the leaves of the three genotypes (Figure 3.10). This might be associated with the increase observed in the availability of its precursors (cysteine, glutamate and glycine) (Appendix 3.1). After GLP treatment it was detected an increase in GSSG content, which can be related to the increase in lipid peroxidation detected in this organ after this herbicide (Figure 3.9). Accordingly, the ratio GSH/GSSG was not modified after GLP treatment in leaves of wild-type plants as has been reported before in soybean leaves (Vivancos et al., 2011). IMX provoked an increase in the GSH/GSSG ratio in the leaves of the three genotypes, suggesting different effects of both herbicides on the redox status. Indeed, redox status in roots was more affected

after GLP treatment than after imazethapyr treatment (Zabalza et al., 2004; Orcaray et al., 2012).

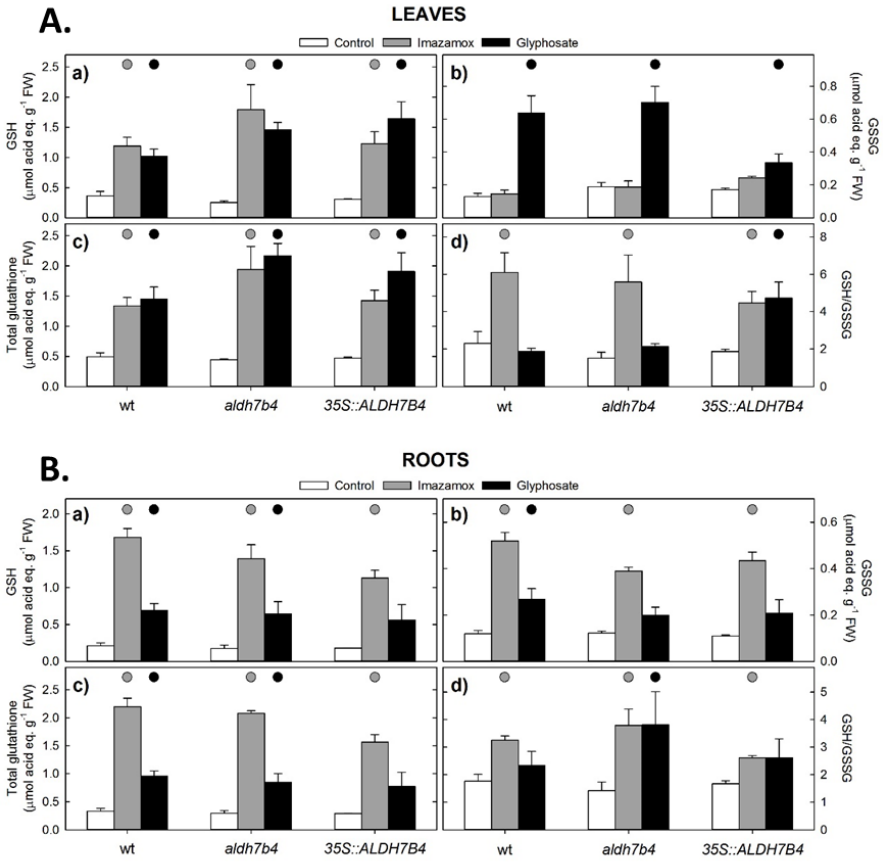


Figure 3.10. Reduced glutathione content (GSH) (a), oxidized glutathione content (GSSG) (b), sum of GSH and GSSG (c) and ratio GSH/GSSG (d) in the leaves (A) and the roots (B) of wild-type (wt) *Arabidopsis thaliana* Col-0 plants, *aldh7b4* mutants and *35S::ALDH7B4* mutants, untreated (control) or treated with imazamox or glyphosate for 3 days. Values represent the mean ± SE (n = 5 biological replicates). Significant variations are marked with ● for differences between control and imazamox-treated plants, and with ● for differences between control and glyphosate-treated plants (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).

In roots, similarly to leaves, total glutathione and GSH contents increased after both herbicide application in wild-type and *aldh7b4* mutants (Figure 3.10). This pattern was also detected in the roots of *35S::ALDH7B4* plants after IMX

but not after GLP treatment. The overexpression of *ALDH7B4* abolished the changes in the glutathione contents induced by GLP in roots.

Altogether, the results presented here indicate that the lack or the overexpression of the *ALDH7B4* modifies the response of the plants to GLP but not to IMX. Differences between the two organs were also detected. In leaves, GLP application provoked an oxidative stress (since MDA and GSSG contents were increased) but it is not related to *ALDH7B4* as the same pattern was detected in the leaves of the mutant plants. In roots, no oxidative stress was detected after GLP treatment (since the levels of MDA and GSSG did not increase) in the three studied genotypes. However, the increase in total glutathione and GSH were abolished in plants overexpressing *ALDH7B4.* The increase in total glutathione pool detected in soybean after GLP treatment was suggested to allow glutathione-dependent GLP detoxification pathways (Vivancos et al., 2011). In this context, it can be hypothesized that the ALDH7B4 can contribute to the detoxification of this herbicide.

In conclusion, the results included in this section show that the role of ALDH7B4 is not involved in alleviating oxidative stress after herbicide treatment.

b) ALDH7B4 alleviates the physiological effects of ABIHs on root carbon metabolism

To further study the role of ALDH7B4, the physiological characterization was done in wild-type and mutant *Arabidopsis* lines, by evaluating the previously described common effects of ABIHs. Specifically, the effects of ABIHs on the *in vitro* activities of the enzymes involved in the ethanol fermentation, the free amino acid content, the soluble sugars and starch content and the organic acid content were monitored in the leaves and the roots of *A. thaliana* plants after three days of treatment with IMX or GLP. Since the effects of IMX and GLP on *A. thaliana* wild-type plants have been shown and discussed

in the Chapter 1 of this thesis, a summary of these effects is presented in the Table 3.6. The original data are presented in the Appendix 3.

		LEAVES		ROOTS	
		IMX	GLP	IMX	GLP
Growth		↓	↓	↓	↓
Ethanol fermentation	*In vitro* activities of PDC and ADH	↑	↑	↑	↑
Free amino acids	Total free amino acids	↑	↑	↑	↑
	% of BCAA	↓	↑	↓	↑
	% of AAA	=	↑	↓	=
	% acidic amino acids	↓	↓	↓	↓
	% amide amino acids	=	↑	↑	=
Carbohydrate content	Soluble sugars	↑	↑	↑	↑
	Starch	↑	↑	=	↑
Organic acids	Pyruvate	=	↑	↑	↑
	Lactate	=	↑	↓	↓
	α-ketoglutarate, succinate, malate	↑	↑	↑	=
	Citrate	↓	↓	↑	=
	Shikimate	=	↑	=	↑
	Quinate	=	↑	↑	=

Table 3.6. The prominent physiological effects provoked by imazamox or glyphosate application (after three days) in the leaves and the roots of *Arabidopsis thaliana* wild-type plants. =, ↑ and ↓ indicate no change, increase or decrease of the corresponding parameter after herbicide treatment comparing to the effects found in the untreated plants, respectively. AAA, aromatic amino acids; ADH, alcohol dehydrogenase; BCAA, branched-chain amino acids; IMX, imazamox; GLP, glyphosate; PDC, pyruvate decarboxylase; TCA, tricarboxylic acid.

After performing the full characterization of the physiological profile in the three plant lines, significant differences between the mutants and wild-type plants were detected for some parameters. All the results are included in the Appendix 3. Figure 3.11 shows the parameters where differences between the herbicide-treated wild-type and the *aldh7b4* or *35S::ALDH7B4* mutants were found.

More differences were detected in the roots than in the leaves (Figure 3.11). Most of the differences that will be discussed are not significant but they will be mentioned in this study due to its physiological relevance in the toxicity of the herbicides.

Regarding the total free amino acid content, the leaves of GLP-treated *aldh7b4* mutants presented higher levels comparing to the leaves of the GLP-treated wild-type plants (Figure 3.11.a). By contrast, the total free amino acid content accumulation detected in the roots of the plants overexpressing the *ALDH7B4* gene was slightly less pronounced than the accumulation found in the roots of wild-type plants, after both ABIH application (Figure 3.11.b).

As for the carbohydrate content, the total soluble sugar accumulation found after IMX or GLP treatment in the leaves of the *35S::ALDH7B4* mutants was much lower than the accumulation found in the leaves of wild-type plants, and the same occurred in the leaves of *aldh7b4* mutants after GLP treatment (Figure 3.11.c). Moreover, the total soluble sugars were not accumulated as a consequence of ABIH application in the roots of both studied mutant plants (Figure 3.11.d). The starch content accumulation found in the leaves of IMX-treated *35S::ALDH7B4* mutants was lower than the accumulation found in the leaves of wild-type plants (Figure 3.11.e). Moreover, no starch accumulation was found in the roots of GLP-treated mutants, contrary to what it was found in the wild-type plants (Figure 3.11.f).

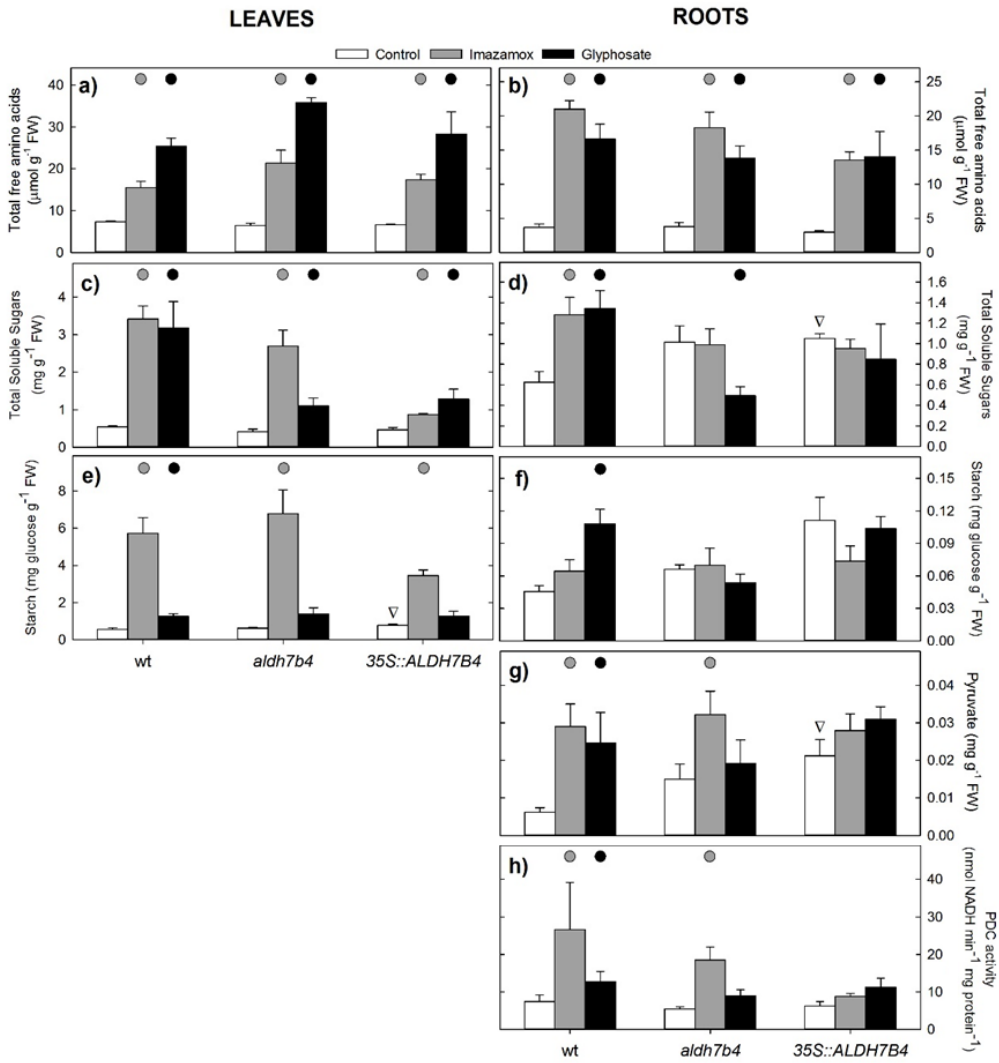


Figure 3.11. Parameters where differences between the herbicide-treated wild-type (wt) *Arabidopsis thaliana* and the *aldh7b4* or *35S::ALDH7B4* mutants were found. The total free amino acid content (a and b), the total soluble sugars content (c and d), the starch content (e and f) in the leaves and the roots, the pyruvate content in the roots (g) and the activity of pyruvate decarboxylase (PDC) in the roots (h) are presented. ∇ indicates differences between the untreated plants of the corresponding genotype and the untreated wt plants (*t*-Test, $p < 0.05$). Values represent the mean ± SE (n = 5 biological replicates). Significant variations are marked with ● for differences between control and imazamox-treated plants, and with ● for differences between control and glyphosate-treated plants (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).

Lastly, no pyruvate accumulation was found in the roots of herbicide-treated mutants, except for the IMX-treated *aldh7b4* mutants (Figure 3.11.g). PDC induction detected in roots of wild-type plants treated with GLP or IMX was abolished in the roots of the plants overexpressing the *ALDH7B4* gene (Figure 3.11.h).

These results show that the carbohydrate and pyruvate accumulation and PDC induction detected in roots of wild-type plants were attenuated in the roots of plants overexpressing the *ALDH7B4* gene, evidencing an attenuation of the typical effects of ABIHs on carbon metabolism. It seems that the increase in the capacity to metabolize the acetaldehyde could help the plant to survive the stress provoked by herbicide application, because this pathway can help to metabolize the accumulated pyruvate after the inhibition of AHAS or EPSPS, or by detoxifying the acetaldehyde produced during ethanol fermentation. Indeed, in *A. thaliana*, this gene has been found to contribute to the survival of the plants to different stress conditions (such as, drought and salinity) (Kotchoni et al., 2006). Moreover, members of the ALDH7 family have also been observed to contribute to different stress tolerance in soybean (Rodrigues et al., 2006) and wheat (Chen et al., 2015).

The results showed that there were not significant differences in the total or individual free amino acid content, which is one of the most important physiological effects provoked by IMX or GLP application in plants. Although the high increase in the expression of the *ALDH7B4* gene after ABIH treatment suggests an important role of this gene in plants upon herbicide treatment, no significant differences were found in the parameters on the nitrogen metabolism between the effects on plants lacking or overexpressing the *ALDH7B4* gene and the effects on the wild-type plants. The decrease in the effects provoked by herbicides detected in plants overexpressing the *ALDH7B4* gene was limited to roots and referred only to carbon metabolism.

If the physiological disturbances on carbon metabolism of ABIHs on roots were slightly alleviated in *ALDH7B4* overexpressing plants, the contrary

behaviour could be expected in plants lacking this gene. Nevertheless, almost no changes were detected in *aldh7b4* mutants. The absence of differences could be related to the fact that other pathways might be activated to compensate the lack of the *ALDH7B4* and/or that other ALDHs could be complementing the absence of the ALDH7B4.

c) PDH-bypass and *de novo* fatty acid synthesis in plants upon ABIH application

As it has been previously presented, ethanol fermentation is induced in plants treated with ABIHs. An induction of the PDH-bypass could therefore be expected as a consequence of ABIH application to metabolize the acetaldehyde produced in the ethanol fermentation. However, no induction of the expression of *ALDH2B4* was detected in IMX- or GLP-treated plants (Figure 3.3 and Figure 3.4). To our knowledge, only members of the family 2 ALDHs have been investigated for their participation in the PDH-bypass pathway in *A. thaliana* (Wei et al., 2009), and no information is available with respect to the participation of other ALDHs.

Contrary to what it has been observed for other stresses, our study revealed that the ALDH7B4 is not related to an attenuation of oxidative stress upon ABIH treatment, and thus, it is probably involved in another process, perhaps in the PDH-bypass. Indeed, in rice, acetaldehyde has been shown to be metabolized by members of the family 7 ALDHs (Shin et al., 2009). Previous results of this section have shown that the toxicity of the herbicides is slightly alleviated in the roots of the plants overexpressing the *ALDH7B4* (Figure 3.11). In this context, we tested whether the ALDH7B4 is involved in the PDH-bypass in plants treated with ABIHs and if that could be the reason for the detected attenuation of the physiological effects provoked by the herbicides.

In the Chapter 1 of the present thesis, a mutant line defective for the *ADH1* was used to check the role of the induction of the fermentative pathway in the toxicity of the herbicides. Interestingly, the results showed that the effects provoked by the herbicides were slightly attenuated in *A. thaliana* plants lacking

fermentation, although the lethality of the herbicides did not change significantly. If the ALDH7B4 participates in the PDH-bypass, the effect of decreasing the acetaldehyde consumption by ADH in the *adh1* mutants or increasing acetaldehyde consumption by *ALDH7B4* in the *35S::ALDH7B4* mutants could be considered similar or parallel, because both of them would fuel pyruvate consumption through the PDH-bypass. Interestingly, contrary to the wild-type plants, pyruvate did not accumulate in the roots of mutant plants overexpressing the *ALDH7B4* (Figure 3.11) or lacking *ADH1* (Chapter 1, Figure 1.37) upon ABIH treatment. This abolishment of pyruvate accumulation suggests that acetaldehyde is fuelled to the PDH-bypass and that ALDH7B4 is metabolizing the acetaldehyde produced from pyruvate. Indeed, acetaldehyde is produced in the cytosol and due to its toxicity it is likely to be metabolized in this cell compartment by ALDH7B4 or other cytosolic ALDHs. Considering the expected metabolic and the detected physiological similarities in the alleviation of the toxicity of ABIHs between the *35S::ALDH7B4* and *adh1* mutant lines, the *adh1* transgenic line has also included in this section.

In the PDH-bypass, the acetaldehyde produced during the ethanol fermentation is metabolized by ALDHs to produce acetate which is converted into acetyl-CoA by the ACS (Pronk et al., 1994). To evaluate whether the PDH–bypass is induced in plants upon ABIH treatment the expression pattern of *ACS* was measured in the leaves and the roots of *A. thaliana* plants treated with IMX or GLP (Figure 3.12).

A new common effect of IMX and GLP was detected in the leaves and the roots of treated plants: an increase in the transcription of the *ACS* gene. The upregulation of this gene suggests that ACS converts acetate into acetyl-CoA in the plastids. The acetate is obtained from acetaldehyde, which is synthesized in the cytosol. Since ACS has been described to be located in the plastids, two explanations are possible: the conversion of acetaldehyde into acetate takes place in the cytosol and then acetate is imported from the cytosol to the plastids, or the conversion of acetaldehyde into acetate takes place in the plastids.

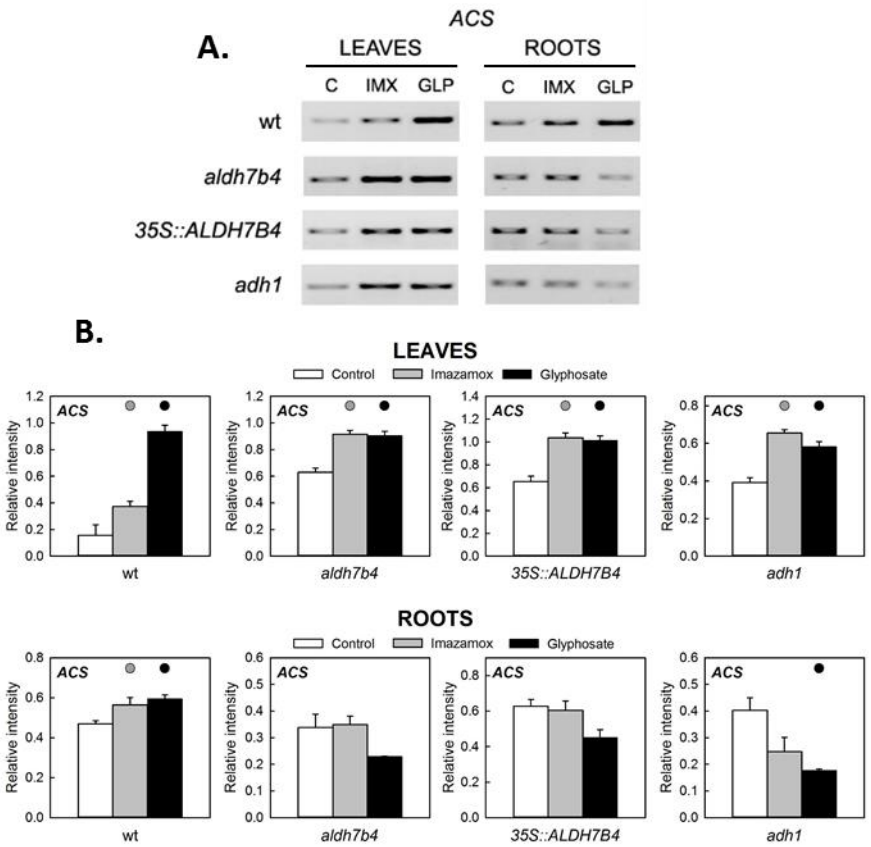


Figure 3.12. Expression pattern of *ACS* in the leaves and the roots of wild-type (wt) *Arabidopsis thaliana* Col-0 plants, *aldh7b4* mutants, *35S::ALDH7B4* mutants and *adh1* mutants, untreated (C) or treated with IMX or GLP for 3 days. **A.** Gels shown are representative examples of the six RT-PCRs carried out. **B.** The relative band intensity (*GENE OF INTEREST* / *ACTIN2*) measured by the Quantity One software (Bio-Rad Laboratories Inc., Hercules, CA, USA). Values represent mean ± SE (n=6). ● and ● indicate significant difference between control and IMX- or GLP-treated plants, respectively (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$). C, control; GLP, glyphosate; IMX, imazamox.

However, it is very unlikely that acetaldehyde would freely traverse the membrane due to its reactivity and potential toxicity. The expression of the *ALDH3I1,* a gene coding for the only known plastidial ALDH in *A. thaliana*, was not affected by the presence of the herbicides (Figure 3.3 and Figure 3.4). Moreover, the expression of the *ALDH2B4*, which encodes a mitochondrial

enzyme known to take part in the PDH-bypass in *A. thaliana*, was not affected by ABIH application. The only *ALDH* upregulated by both herbicides in both organs was the *ALDH7B4*, suggesting a role for the ALDH7B4 in the cytosolic conversion of acetaldehyde into acetate.

To confirm that the conversion of the acetaldehyde coming from the induced PDC into acetate was catalysed by the ALDH7B4, an experiment was performed with a double *pdc1-pdc2* mutant. The use of this mutant offered the opportunity of assessing the physiological role of ALDH7B4 with an expected lower availability of acetaldehyde, as this mutant lacks the two predominant pyruvate-consuming *PDC* genes. This mutant line was compared to the wild-type plants. Seedlings of *A. thaliana* (wild-type and *pdc1-pdc2* mutants) were grown under sterile conditions and were treated with IMX or GLP for 5 days. The transcript levels of the *ALDH7B4* and *ACS* genes were measured by qPCRs (Figure 3.13).

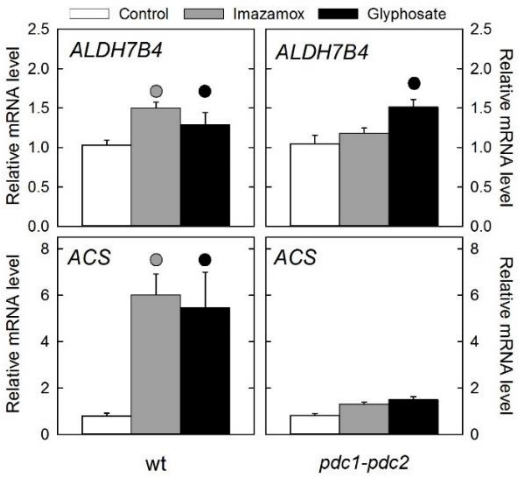


Figure 3.13. Relative expression levels (transcripts) of the *ALDH7B4* and *ACS* genes in imazamox- or glyphosate-treated (for five days) wild-type (wt) *Arabidopsis thaliana* Col-0 and *pdc1-pdc2* seedlings grown under sterile conditions. Values represent mean ± SE (n=5). ● and ● indicate significant difference between control and imazamox- or glyphosate-treated plants, respectively (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).

In wild-type seedlings, the expression of *ALDH7B4* was induced after both ABIH treatments, as it was detected in the plants grown under non-sterile hydroponic system (Figure 3.13). Additionally, both herbicides provoked an increase in the expression of the *ACS*. The concomitant increases in the expression of *ALDH7B4* and *ACS* after ABIH treatment supports an induction of the PDH-bypass by the herbicides.

While the increase of expression of the *ALDH7B4* upon IMX treatment was abolished in the *pdc1-pdc2* seedlings, the expression of the *ALDH7B4* also increased in the GLP-treated *pdc1-pdc2* seedlings (but not as much as in the wild-type seedlings), indicating that the acetaldehyde used as substrate by ALDH7B4 is not only coming from PDC1 or PDC2 (it could be synthesized by PDC3 and/or PDC4). Interestingly, the induction of *ACS* observed in wild-type plants after both ABIH applications was abolished in the *pdc1-pdc2* double mutants. The simultaneous decrease in the expression of the enzymes in the *pdc1-pdc2* mutants supports the hypothesis that the PDH-bypass is induced in ABIH-treated plants, as an alternative pathway for pyruvate consumption. Moreover, the results suggest that ALDH7B4 can be participating in that pathway (at least in the case of IMX), because almost no induction of the expression of this gene is observed in the *pdc1-pdc2* mutants after herbicide application, suggesting that the ALDH7B4 metabolizes the acetaldehyde synthesized from pyruvate via PDC.

Regarding the expression of *ACS* after ABIH application in the *aldh7b4,* the *35S::ALDH7B4* and the *adh1* mutants (Figure 3.12), it was detected that the notorious increase in the expression of *ACS* detected in the leaves of ABIH-treated wild-type plants was attenuated in all the studied mutant lines, even though the decrease was not significant.

As for the roots, the upregulation of the *ACS* gene detected in the wild-type plants was attenuated in the *aldh7b4* mutants and abolished in the *35S::ALDH7B4* and *adh1* mutants. Indeed, the expression of *ACS* was downregulated after GLP treatment in the roots of the mutant plants (although

the decrease was only significant after GLP in the roots of *adh1* plants) (Figure 3.12). Interestingly, contrary to the wild-type plants, pyruvate did not accumulate (Figure 3.11.g) and the PDC activity was less induced in the roots of mutant plants overexpressing the *ALDH7B4* (Figure 3.11.h) or lacking the *ADH1* (Chapter 1, Figure. 1.22 and Figure 1.37) upon ABIH treatment. In this context, it can be suggested that the lower increase in ACS expression is related to a lower availability of pyruvate and the concomitant lower flux through PDC activity.

In the plastids, the acetyl-CoA is the precursor for *de novo* fatty acid biosynthesis (Figure 3.2). In the first reaction the acetyl-CoA is metabolized by the ACC2. Fatty acids are grown by sequential condensation of two-carbon units by enzymes of the fatty acid synthase complex. During the first turn of the cycle, the condensation reaction is catalysed by ketoacyl-ACP synthase (KAS) III. For the next six turns of the cycle, the condensation reaction is catalysed by isoform I of KAS. Finally, KAS II is used during the conversion of 16:0 to 18:0 (Li-Beisson et al., 2013). The fatty acid content and the expression pattern of different genes involved in the *de novo* fatty acid biosynthesis (*ACC2*, *KASIII*, *KASI, KASII*) were measured, to evaluate if the acetyl-CoA is fuelled to the *de novo* fatty acid biosynthesis.

The total content of fatty acids (Figure 3.14) and the expression pattern of the genes coding for the main enzymes catalysing the synthesis of fatty acids (*ACC2*, *KASIII*, *KASI*) are shown in Figure 3.15 for the leaves and Figure 3.16 for the roots.

In general, the total fatty acid content was not significantly affected by the ABIH treatment, although some patterns can be proposed. In the leaves of the wild-type plants (Figure 3.14), a minor decrease in the total fatty acid content was detected after both herbicide application, even though the expression of the genes that participate in *de novo* fatty acid biosynthesis (*ACC2*, *KASI* and *KASIII)* was not generally affected by the herbicides in the wild-type plants (Figure 3.15). The sole changes detected in the leaves of wild-type plants were

an increase in the expression of *KASIII* following IMX application and a decrease in the expression of *KASI* after treatment with GLP. Regarding the *aldh7b4* mutants, while the total fatty acid content present in the leaves was very similar to the one present in the wild-type plants (Figure 3.14), the expression of the *KASIII* changed since both herbicides downregulated this gene (Figure 3.15). Interestingly, a significant decrease in the total fatty acid content was detected in the leaves of both *ALDH7B4* overexpressing and *adh1* mutants after GLP application (Figure 3.14). This decrease was related to a decrease in the expression of the genes involved in the *de novo* fatty acid biosynthesis, especially with the *ACC2* (Figure 3.15).

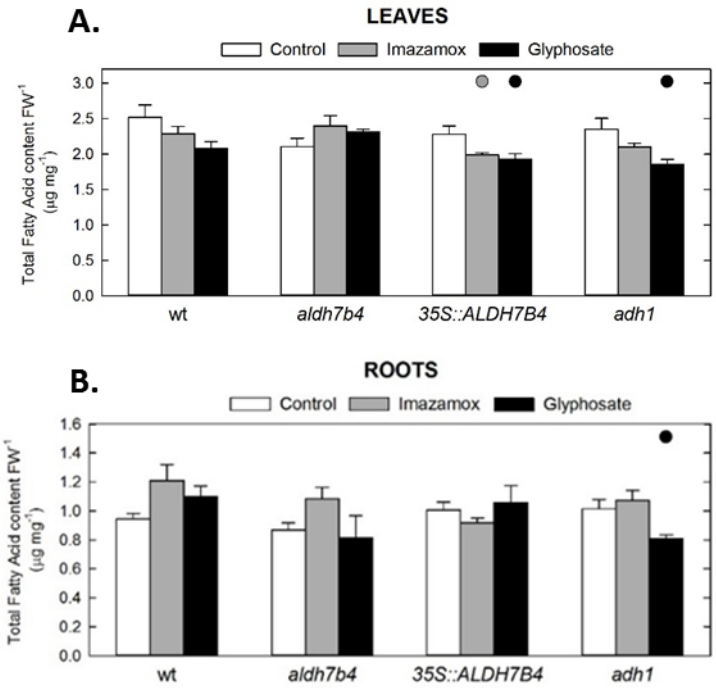


Figure 3.14. Total fatty acid content in the leaves (A) and the roots (B) of wild-type (wt) *Arabidopsis thaliana* Col-0 plants, *aldh7b4* mutants, *35S::ALDH7B4* mutants and *adh1* mutants, untreated (control) or treated with imazamox or glyphosate for 3 days. Values represent mean ± SE (n=5). ○ and ● indicate significant difference between control and imazamox- or glyphosate-treated plants, respectively (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).

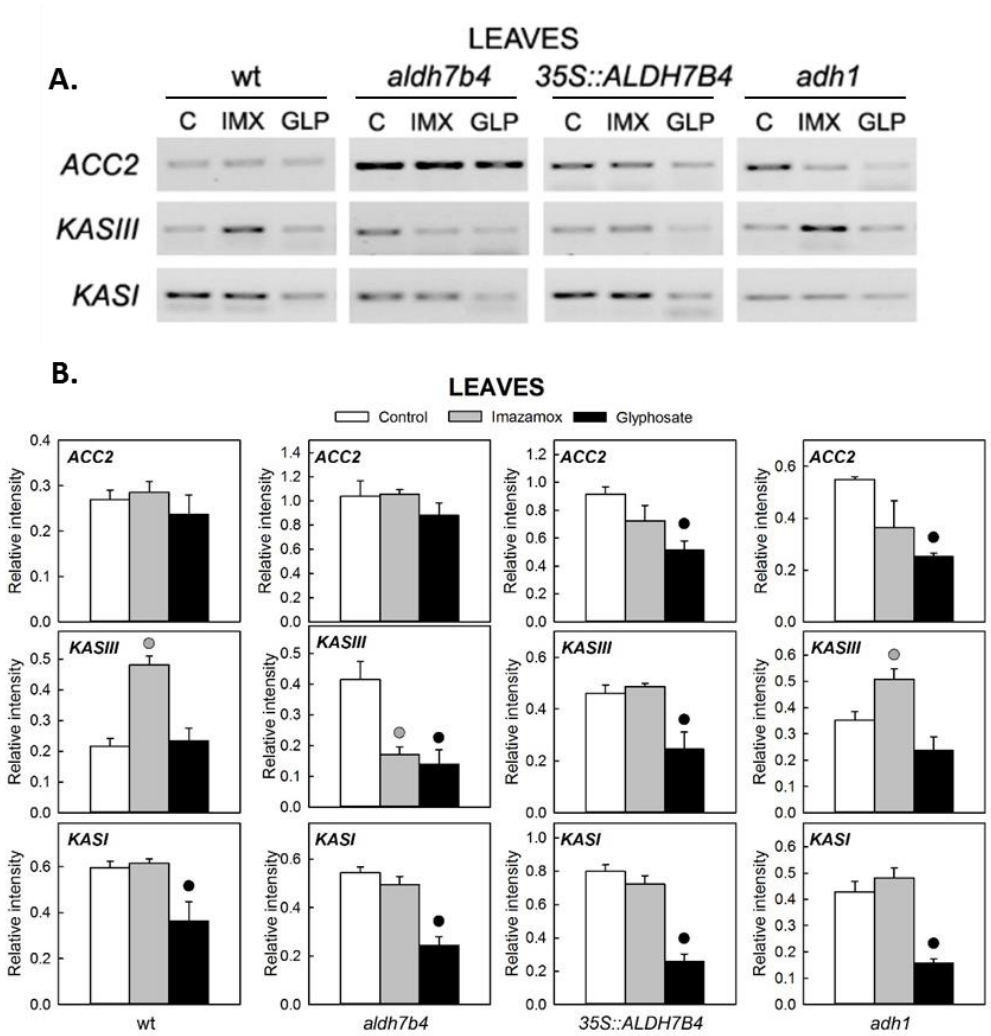


Figure 3.15. Expression pattern of *ACC2, KASIII and KASI* in the leaves of wild-type (wt) *Arabidopsis thaliana* Col-0 plants*, aldh7b4* mutants, *35S::ALDH7B4* mutants and *adh1* mutants, untreated (control) or treated with IMX or GLP for 3 days. **A.** Gels shown are representative examples of the six RT-PCRs carried out. **B.** The relative band intensity (*GENE OF INTEREST*/ *ACTIN2*) measured by the Quantity One software (Bio-Rad Laboratories Inc., Hercules, CA, USA). Values represent mean ± SE (n=6). ◌ and ● indicate significant difference between control and IMX- or GLP-treated plants, respectively (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$). C, control; GLP, glyphosate; IMX, imazamox.

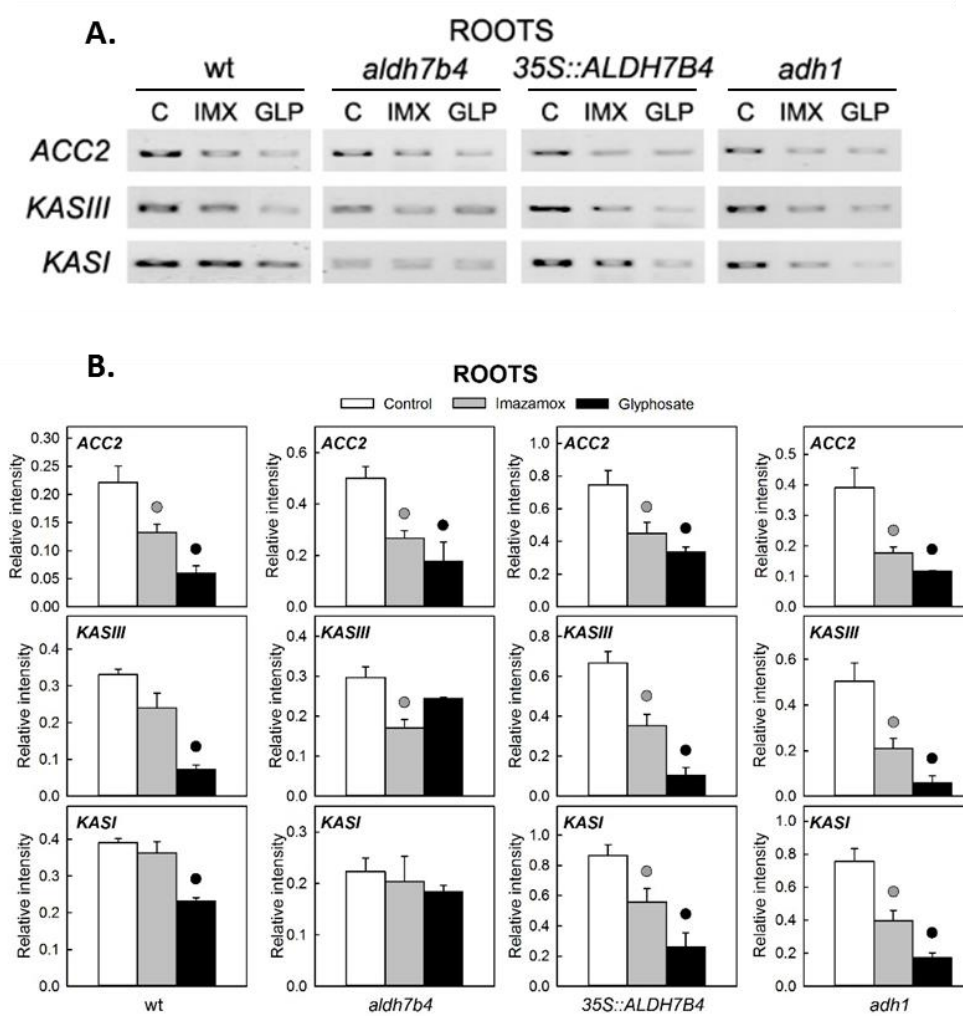


Figure 3.16. Expression pattern of *ACC2, KASIII and KASI* in the roots of wild-type (wt) *Arabidopsis thaliana* Col-0 plants, *aldh7b4* mutants, *35S::ALDH7B4* mutants and *adh1* mutants, untreated (control) or treated with IMX or GLP for 3 days. **A.** Gels shown are representative examples of the six RT-PCRs carried out. **B.** The relative band intensity (*GENE OF INTEREST*/*ACTIN2*) measured by the Quantity One software (Bio-Rad Laboratories Inc., Hercules, CA, USA). Values represent mean ± SE (n=6). ● and ● indicate significant difference between control and IMX- or GLP-treated plants, respectively (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$). C, control; GLP, glyphosate; IMX, imazamox.

Contrary to the leaves, in the roots of the wild-type plants a minor increase in the total fatty acid content was detected after ABIH application (Figure 3.14), even though the expression of the *ACC2*, *KASIII* and *KASI* genes decreased as a consequence of ABIH application, being the GLP the herbicide that provoked higher decrease (Figure 3.16). The total fatty acid content found in the roots of the three studied mutants was similar to the one found in the wild-type plants, with the exception that a decrease in this parameter was found in the roots of the GLP-treated *adh1* mutants (Figure 3.14). The expression pattern of the genes involved in the *de novo* fatty acid biosynthesis found in the roots of the *35S::ALDH7B4* and *adh1* mutants was the same as the one observed in the wild-type plants, and in these mutants the effect of IMX was high and provoked a significant decrease in the expression of *KASIII* and *KASI* genes (Figure 3.16). By contrast, in the *aldh7b4* mutants, the effect of herbicides on the expression of *KASIII* and *KASI* was attenuated (Figure 3.16).

These results discard the proposed hypothesis about the acetyl-CoA being fuelled for the *de novo* biosynthesis of fatty acids in plants treated with ABIHs. Although an important increase in the acetyl-CoA pool would be expected in the leaves and roots by the concomitant increase in the activity of PDC (Figure 3.11) and the transcript levels of *ALDH7B4* (Figure 3.3 and Figure 3.4) and *ACS* (Figure 3.12) upon ABIH treatment, the decrease in the total fatty acid content suggests that the produced acetyl-CoA is not fuelled to the *de novo* fatty acid biosynthesis. Instead, this metabolite seems to be fuelled to other pathways (TCA cycle in the mitochondria, glyoxilate cycle in the glyoxysomes, and fatty acid elongation and secondary metabolite biosynthesis in the cytosol) (Figure 3.1). However, acetyl-CoA has a limited permeability and no membrane transporters have been found in plants, thus, it seems that these metabolite needs to be synthetized within the compartment where it would be metabolized.

Plastidic acetyl-CoA is synthesized by two enzyme systems, the PDH complex and ACS. The existence of ACS was discovered first (Smirnov, 1960)

and for years it was considered a primary source of acetyl-CoA for lipid biosynthesis. The discovery of PDH complex provided a more direct connection between acetyl-CoA formation and central metabolism (Reid et al., 1977), but it still took some years to clarify that the PDH complex is the major source of acetyl-CoA for fatty acid formation in the plastids (Oliver et al., 2009).

Although the acetyl-CoA pool generated by ACS from acetate seems redundant for fatty acid biosynthesis, ACS is hypothesized to play a specialized role in certain cells and tissues (Ke et al., 2000). Our results suggest that acetyl-CoA levels would be limiting to fatty acid synthesis probably due to an affected PDH complex. Although the specific effects of ABIHs on PDH complex have not been studied yet, it can be hypothesized a negative effect of the ABIHs on this complex, because carbon metabolism has been described to be notoriously affected by ABIHs (Zabalza et al., 2004; Orcaray et al., 2012).

d) Pattern of individual fatty acids in plants after ABIH treatment

The effect of herbicides on the percentage of the individual fatty acid content was analysed in the leaves and the roots of *A. thaliana* wild-type plants and the *aldh7b4*, *35S::ALDH7B4* and *adh1* mutants (Figures 3.18 and 3.19).

Regarding the leaves of wild-type plants, the effect of IMX was not so high and it just provoked a slight increase in the percentage of 16:2, and a mild decrease in the percentage of 18:0, 16:3 and 18:1. By contrast the effect of GLP was higher and it provoked an accumulation of the 16:0, 16:1, 16:2, 18:1 and 18:2 fatty acids and a decrease in the 16:3 and 18:3 fatty acids (Figure 3.18).

Interestingly, the effects of ABIHs on the fatty acid content in the leaves of both *aldh7b4* and *35S::ALDH7B4* mutants was very similar, and they presented the same pattern observed in the wild-type plants. Although the effect of herbicides on these mutants was alleviated comparing to the effect provoked in the wild-type plants (Figure 3.18).

By contrast, in the *adh1* mutants, there were some differences regarding the effects of herbicides on the leaf fatty acid content (Figure 3.18). While in the wild-type plants the percentage of 16:1 and 18:2 increased after GLP application and the percentage of 18:3 decreased, the opposite effect was observed in the *adh1* mutants and the percentage of 16:1 and 18:2 decreased and the percentage of 18:3 increased as a consequence of GLP application. Moreover, while in the wild-type plants GLP provoked an increase in the percentage of 16:2 and a decrease in the percentage of 16:3, in the *adh1* mutants, the percentage of these two fatty acids did not change with respect to the untreated plants (Figure 3.18).

As it occurred in the leaves, GLP affected more the fatty acid content in the roots of wild-type plants (Figure 3.19). The percentage of the 14:0, 16:1, 16:3 and 18:1 increased as a consequence of IMX application and, by contrast the percentage of 16:0 decreased. On the other hand, GLP provoked an increase in the percentage of 14:0, 16:0 and 18:1, while it provoked a decrease in the 18:3 content.

The three studied mutant lines presented a similar pattern in the percentage of the individual fatty acids to the one found in the roots of wild-type plants, and just few dissimilarities were found. The effect of GLP in the *aldh7b4*, *35S::ALDH7B4* and *adh1* mutants was higher, and the increase in the percentage of 16:0 and the decrease in the percentage of 18:3 detected in the mutant lines were more prominent than in the wild-type plants. Moreover, while the percentage of 18:2 did not change as a consequence of GLP application in the wild-type plants, in the *aldh7b4* mutants a decrease was found. On the other hand, while no change in the percentage of 18:3 was found in the IMX-treated wild plants, a decrease on this parameter was found in the *35S::ALDH7B4* mutants (Figure 3.16).

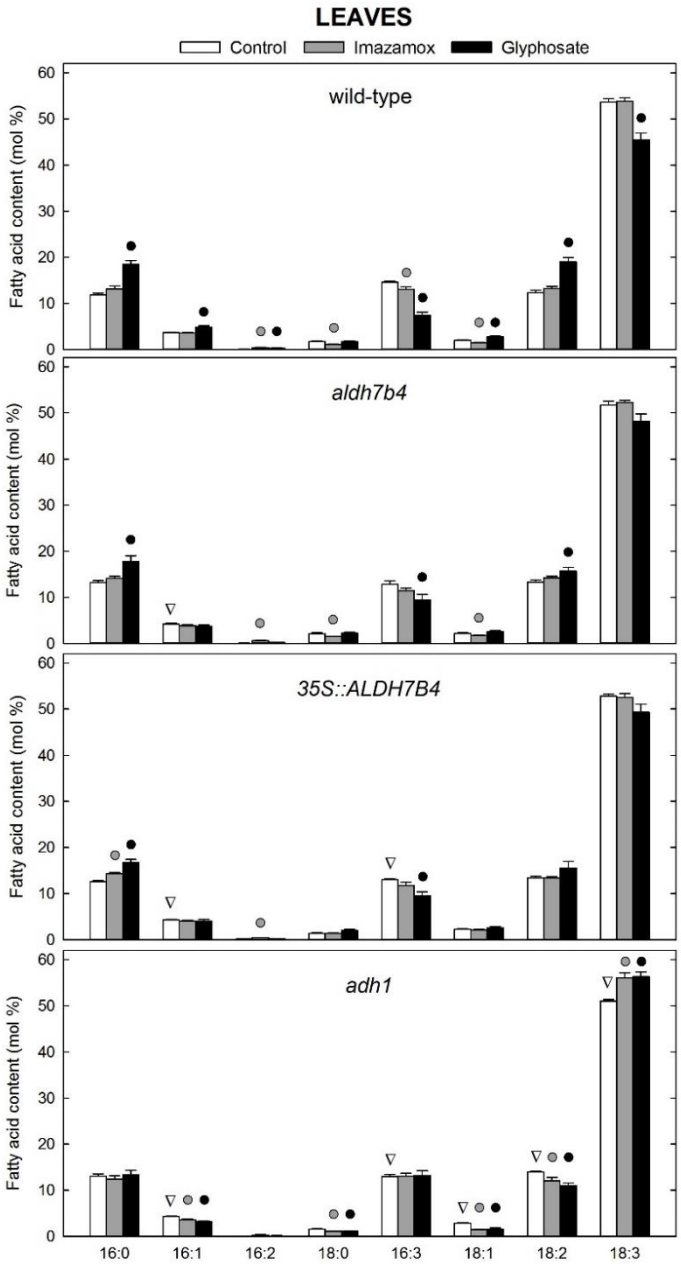


Figure 3.18. Individual fatty acid content (expressed as the percentage of the total fatty acids) in the leaves of wild-type (wt) *Arabidopsis thaliana* Col-0 plants, *aldh7b4* mutants, *35S::ALDH7B4* mutants and *adh1* mutants, untreated (control) or treated with imazamox or glyphosate for 3 days. Values represent mean ± SE (n=5). ∇ indicates differences between the untreated plants of the corresponding genotype and the untreated wt plants (*t*-Test, $p < 0.05$). ● and ● indicate significant difference between control and imazamox- or glyphosate-treated plants, respectively (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).

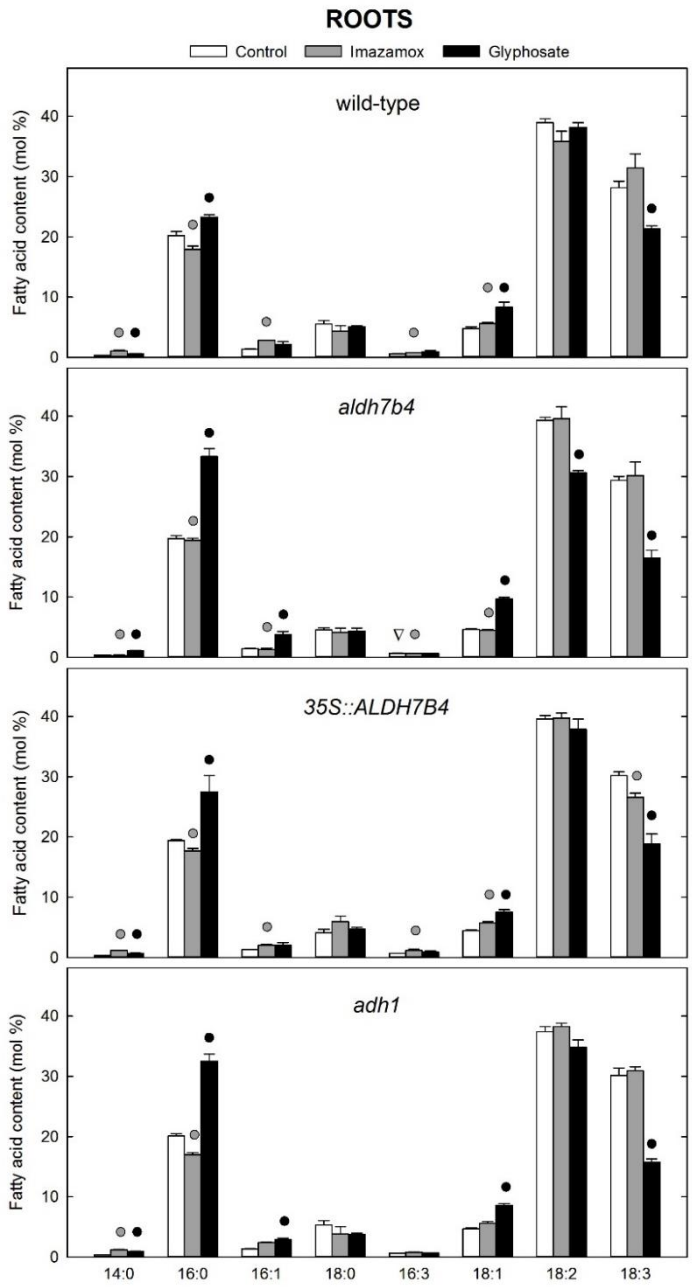


Figure 3.19. Individual fatty acid content (expressed as the percentage of the total fatty acids) in the roots of wild-type (wt) *Arabidopsis thaliana* Col-0 plants, *aldh7b4* mutants, *35S::ALDH7B4* mutants and *adh1* mutants, untreated (control) or treated with imazamox or glyphosate for 3 days. Values represent mean ± SE (n=5). ∇ indicates differences between the untreated plants of the corresponding genotype and the untreated wt plants (*t*-Test, $p < 0.05$). ◌ and ● indicate significant difference between control and imazamox- or glyphosate-treated plants, respectively (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).

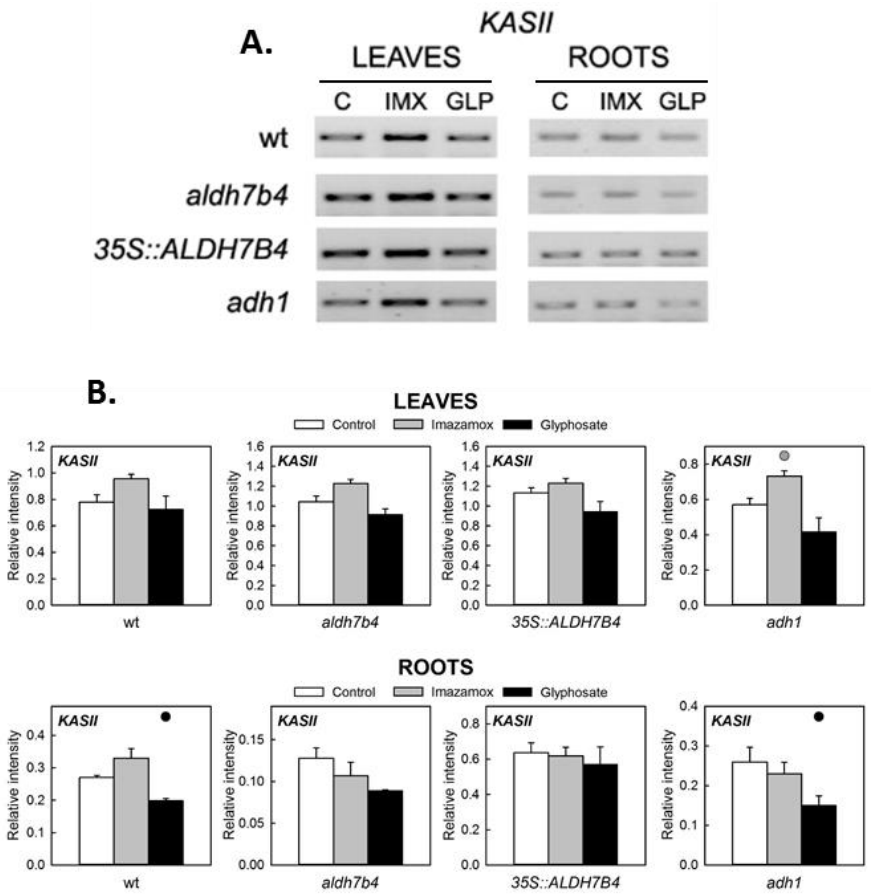


Figure 3.20. Expression pattern of *KASII* in the leaves and the roots of wild-type (wt) *Arabidopsis thaliana* Col-0 plants, *aldh7b4* mutants, *35S::ALDH7B4* mutants and *adh1* mutants, untreated (C) or treated with IMX- or GLP- for 3 days. **A**. Gels shown are representative examples of the six RT-PCRs carried out. **B.** The relative band intensity (*GENE OF INTEREST* / *ACTIN2*) measured by the Quantity One software (Bio-Rad Laboratories Inc., Hercules, CA, USA). Values represent mean ± SE (n=6). ● and ● indicate significant difference between control and IMX- or GLP-treated plants, respectively (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$). C, control; GLP, glyphosate; IMX, imazamox.

The study of the individual fatty acids content revealed that the two herbicides have a different effect on this parameter (Figure 3.18 and Figure 3.19). Moreover, as the patterns were similar between the wild-type plants, the *aldh7b4* and *35S::ALDH7B4* mutants regarding the percentage of the individual fatty acids, it seems that the ALDH7B4 does not affect the fatty acid composition. By contrast, the percentage of the individual fatty acids observed

in the leaves of the *adh1* mutants was different to the wild-type plants suggesting that the ADH1 could have a role in the fatty acid composition (Figure 3.18).

The fact that the percentage of 16:0 fatty acids increases and of 18:3 decreases as a consequence of GLP application suggests that the synthesis of 18:3 fatty acids from the precursor 16:0 is disrupted. To evaluate this, the expression of the *KASII,* a gene encoding one of the enzymes that participates in the biosynthesis of 18:3 fatty acids from 16:0, was monitored in the leaves and the roots of ABIH-treated plants (Figure 3.20). The results showed that, while IMX almost did not affect the expression of this gene, a decrease in the transcript levels of *KASII* was detected as a consequence of GLP application. Thus, it seems that the detected decrease in the 18:3 content is due to a reduced synthesis rate. Nevertheless, a degradation of 18:3 could also occurred contributing to the decrease in the 18:3 content. The 18:3 is a substrate for the jasmonic acid and many other oxylipins. A connection between the 18:3 content and the expression of *ALDH7B4* has been proposed upon wounding, since it has been observed that in the triple mutant *fad3- 2fad7-2fad8*, which does not accumulate 18:3, the expression of the *ALDH7B4* was impaired (Berger, 2002; Matsui, 2006). A regulatory pathway for the expression of the gene *ALDH7B4* upon wounding has been proposed recently whereby the oxylipins derived from linolenic acid (18:3) may activate the transcription factors responsible for the activation of the expression of the *ALDH7B4* (Missihoun et al., 2014).

3. 5. CONCLUSIONS

In this chapter, new common effects of ABIHs on pyruvate-consuming pathways have been evaluated. The possible effects of ABIHs on different aldehyde dehydrogenases and on the PDH-bypass have been studied.

The main conclusions of this chapter are:

- The study of the effects of ABIHs on the gene expression of selected ALDHs revealed a new common effect in the physiological response to the aromatic or branched-chain amino acid biosynthesis inhibiting herbicides: the induction of the *ALDH7B4* gene expression. This effect was confirmed monitoring the ALDH7B4 promoter activity.
- The induction of *ALDH7B4* after the application of both herbicides does not seem to be related to the detoxification of aldehydes derived from lipid peroxidation.
- The use of a mutant line defective for the *ALDH7B4* gene and a mutant line overexpressing this gene allowed to outline a role of the ALDH7B4 in the toxicity of the herbicides. The induction of the *ALDH7B4* expression seems to alleviate the typical effects of herbicides on carbon metabolism, while it does not affect the effects of herbicides on the amino acid content.
- The upregulation of the *ACS* gene upon herbicide treatment indicates that the PDH-bypass is induced by both herbicides. The use of a *pdc1-pdc2* double mutant allowed to elucidate that after IMX application, the conversion of the acetaldehyde produced by PDC to acetate is being catalysed mainly by the ALDH7B4.
- The total fatty acid content was not affected by herbicides and the expression of the main *de novo* fatty acid synthesizing enzymes (*ACC2*, *KAS III* and *KAS I*) decreased after ABIH application, suggesting a lower availability of the substrate acetyl-CoA, probably due to a decreased production rate by the main biosynthetic enzyme (PDH complex).

- It was not possible to find a common physiological effect of the herbicides on the content of individual fatty acid content because different patterns were found in leaves and roots after one herbicide or the other. One of the most significant effects is that in the roots of GLP-treated plants, the synthesis of 18:3 species from 16:0 is compromised probably due to a downregulation of the *KASII* gene expression.
- The ALDH7B4 does not seem to influence the individual fatty acid composition since both *aldh7b4* and *35S::ALDH7B4* mutants presented the same pattern observed in the wild-type plants regarding the percentage of the individual fatty acid content.

- GENERAL OVERVIEW -

GENERAL OVERVIEW

Herbicide application has become the main method in the control of unwanted plants in modern agriculture. However, the use of herbicides is now threatened by the evolution of herbicide-resistant weeds. To manage this situation, new herbicides with new sites of action are badly needed. Knowing more about the physiological side effects triggered in plants after herbicide application would help to elucidate why plants die as a consequence of herbicide treatment. This piece of knowledge would help in the discovery of new herbicides with new sites of action.

Among the commercially available herbicides, the amino acid biosynthesis inhibiting herbicides (ABIHs) have become the most extensively used ones. In the present thesis, we have focused on the study of the physiological side effects provoked by branched-chain amino acid (BCAA) and aromatic amino acid (AAA) biosynthesis-inhibiting herbicides. These herbicides inhibit the enzyme acetohydroxyacid synthase (AHAS) in the BCAA biosynthesis and the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) in the AAA biosynthesis. Although they target different enzymes, some common physiological effects have been attributed to both types of herbicides.

The hypothesis of this thesis is that even though they inhibit different pathways, both types of herbicides kill the plant by a similar mechanism. Thus, the main objective of this thesis is to gain further insights into the common toxicity of BCAA and AAA biosynthesis-inhibiting herbicides, paying special attention to the pyruvate-consuming pathways that are induced in plants after the application of both types of herbicides.

One common effect triggered in plants treated with BCAA or AAA biosynthesis inhibiting-herbicides is the induction of the ethanol fermentation even though plants are grown under aerobic conditions. This pathway has been described to be induced in plants exposed to several stresses, however, it is not known whether the activation of this pathway upon herbicide treatment helps

the plant to survive the stress provoked by the herbicide or by contrast, it contributes to the plant death. In the Chapter 1 of this thesis, the role of the ethanol fermentation in the toxicity provoked by ABIHs is investigated. For that purpose, two experimental approaches and two plant species were used. On the one hand, it was evaluated if the induction of the ethanol fermentation before the application of a BCAA biosynthesis-inhibiting herbicide modifies the plant response to the herbicide. Pea plants were exposed to low-oxygen conditions before herbicide application so that fermentation was activated. The more characteristic herbicide effects on the plants were compared to the effects provoked in plants not having the ethanol fermentation pathway activated when the herbicide was applied. On the second experimental approach, the physiological effects provoked by one BCAA biosynthesis inhibiting herbicide and one AAA biosynthesis inhibiting herbicide were evaluated on *Arabidopsis thaliana* plants defective for the *ADH1* gene and they were compared with the effects provoked on wild-type plants. Induction of fermentation, free amino acid accumulation and carbohydrate accumulation were used as physiological markers that are provoked by both types of herbicides. In pea plants, it was observed that if the plants presented the ethanol fermentation activated before herbicide application, the effects provoked by the herbicide were alleviated. By contrast, the effects of both types of ABIHs were alleviated in the *A. thaliana* plants lacking the ethanol fermentation. These results indicated that while in pea plants the induction of the ethanol fermentation would reduce the effects provoked by the herbicide, in *A. thaliana*, it would contribute to the toxicity provoked by the herbicide. The role of fermentation in the response of the plants to ABIHs is different for each studied species and cannot be simplified.

The transcriptional regulation of the ethanol fermentation has been deeply studied in plants exposed to low-oxygen conditions. However, no studies evaluating the transcriptional regulation of this pathway upon other stresses have been performed. The general aim of the Chapter 2 of the present thesis, is to gain further insights in the regulation of the ethanol fermentation in plants

treated with ABIHs. For that purpose, pea or *A. thaliana* plants were treated with a BCAA or an AAA biosynthesis inhibiting herbicides, and the regulation of the ethanol fermentation was monitored. In particular, whether the induction of the ethanol fermentation is transcriptionally regulated was evaluated when the plants are treated with ABIHs, and it was observed if it occurs in the same way as in plants exposed to low-oxygen conditions. The results demonstrated that the induction of the ethanol fermentation in plants treated with ABIHs is transcriptionally regulated. However, the results revealed that the transcription factor RAP2.12 is not involved in the transcriptional regulation of this pathway in plants treated with ABIHs. Thus, the mechanism for the induction of this pathway upon ABIH treatment is different to the mechanism that activates the ethanol fermentation in plants exposed to low-oxygen conditions. Additionally, the role of pyruvate (the substrate of PDC) in the regulation of the ethanol fermentation in plants upon ABIH treatment was also evaluated. An increase in the availability of pyruvate could be expected after the application of BCAA and AAA inhibiting herbicides, because they inhibit pyruvate and phosphoenolpyruvate consuming pathways, respectively. The results showed that pyruvate participates in the regulation of the ethanol fermentation, but its role cannot be simply explained by a mimicked effect or a higher substrate availability.

In the Chapter 3 of this thesis, it was investigated whether different aldehyde dehydrogenases (ALDHs) participate in the response triggered by ABIH application in plants. Different members of Family 3 and Family 7 ALDHs have been described to play a role in plants upon different stresses. Besides, members of Family 2 ALDHs have been described to metabolize the acetaldehyde produced during ethanol fermentation. The experiments were performed in *A. thaliana* plants treated with one BCAA and one AAA biosynthesis inhibiting herbicides. The results revealed a new common physiological effect for both ABIHs: the induction of the *ALDH7B4* gene expression in the leaves and the roots of herbicide-treated plants. To elucidate

the role of the ALDH7B4 in the toxicity of ABIHs, two mutant lines were used: one defective for the *ALDH7B4* gene and another one overexpressing the *ALDH7B4* gene. The results revealed that this gene seems to alleviate the stress provoked by the herbicides on carbon metabolism in the roots of treated plants. However, contrary to what it has been described for other stresses, the ALDH7B4 was not related to the detoxification of the aldehydes derived from lipid peroxidation because, in general, it could not be detected an oxidative stress following ABIH application. Additionally, the possible induction of the PDH-bypass in plants treated with ABIHs was investigated. Interestingly, an increase in the *ACS* transcript levels was detected after both ABIH applications, indicating that the induction of the PDH-bypass is another new common effect triggered as a consequence of both ABIH applications.

The total fatty acid content and the expression of the enzymes involved in the *de novo* fatty biosynthesis was evaluated in wild-type plants, the line defective for the *ALDH7B4* gene, the line overexpressing the *ALDH7B4* gene and the line defective for the *ADH1* gene (the same line used in the Chapter 1 of this thesis). However, it could not be established where the acetyl-CoA produced in the PDH-bypass is fueled, because a decrease in the expression of the genes involved in the *de novo* fatty acid biosynthesis was detected in plants after ABIH treatment. The effects of the AHAS and EPSPS inhibiting herbicides on the individual fatty acid content were also monitored and no common pattern could be determined. Moreover, the relative content of the individual fatty acids did not change in the studied mutants indicating that the ALDH7B4 does not seem to affect the fatty acid composition in plants upon ABIH treatment.

In conclusion, the results obtained in this thesis have provided new insights regarding one of the well-known physiological effects triggered by ABIHs: the ethanol fermentation. Additionally, new common physiological effects provoked by these types of herbicides have been revealed (upregulation of the *ALDH7B4* gene expression and induction of the PDH-bypass), supporting the

hypothesis that plant death occurs by similar mechanisms after both types of ABIH applications.

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- APPENDICES -

A. APPENDIX - CHAPTER 1 (PART I)

A two-way analysis of variance (ANOVA) was done to examine the influence of the studied variables (hypoxia and herbicide application) and their possible interaction on the different parameters. An independent analysis was done for each studied day.

1. Ethanol fermentation

1.1 *In vitro* enzymatic activity of PDC

Day 1:

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected model	45356.960[a]	5	9071.392	6.118	.010
Intercept	100144.773	1	100144.773	67.545	.000
Hypoxia	36547.311	2	18273.656	12.325	.003
Herbicide	3317.489	1	3317.489	2.238	.169
Hypoxia x Herbicide	6691.917	2	3345.959	2.257	.161
Error	13343.780	9	1482.642		
Total	145443.901	15			
Corrected Total	58700.740	14			

a. R Squared = .773 (Adjusted R Squared = .646)

Day 3:

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected model	8002.413[a]	5	1600.483	2.674	.076
Intercept	29539.158	1	29539.158	49.357	.000
Herbicide	.149	1	.149	.000	.988
Hypoxia	1443.879	2	721.940	1.206	.333
Hypoxia x Herbicide	4612.658	2	2306.329	3.854	.051
Error	7181.745	12	598.479		
Total	48770.974	18			
Corrected Total	15184.158	17			

a. R Squared = .527 (Adjusted R Squared = .330)

Day 7:

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected model	6302.491[a]	5	1260.498	2.639	.090
Intercept	15973.043	1	15973.043	33.443	.000
Herbicide	3894.417	1	3894.417	8.154	.017
Hypoxia	827.546	2	413.773	.866	.450
Hypoxia x Herbicide	927.936	2	463.968	.971	.412
Error	4776.235	10	477.623		
Total	28803.348	16			
Corrected Total	11078.726	15			

a. R Squared = .569 (Adjusted R Squared = .353)

1.2 *In vitro* enzymatic activity of ADH

Day 1:

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected model	548853.365[a]	5	109770.673	8.126	.003
Intercept	1442189.443	1	1442189.443	106.766	.000
Herbicide	3290.601	1	3290.601	.244	.632
Hypoxia	481332.728	2	240666.364	17.817	.001
Hypoxia x Herbicide	88357.711	2	44178.855	3.271	.081
Error	135079.319	10	13507.932		
Total	2068230.896	16			
Corrected Total	683932.685	15			

a. R Squared = .802 (Adjusted R Squared = .704)

Day 3:

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected model	258755.932[a]	5	51751.186	1.488	.270
Intercept	1284878.183	1	1284878.183	36.941	.000
Herbicide	6141.598	1	6141.598	.177	.682
Hypoxia	160285.273	2	80142.637	2.304	.146
Hypoxia x Herbicide	19858.828	2	9929.414	.285	.757
Error	382602.131	11	34782.012		
Total	2282348.290	17			
Corrected Total	641358.063	16			

a. R Squared = .403 (Adjusted R Squared = .132)

Day 7:

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected model	82479.366[a]	5	16495.873	1.146	.398
Intercept	493359.040	1	493359.040	34.280	.000
Herbicide	26494.335	1	26494.335	1.841	.205
Hypoxia	24839.107	2	12419.553	.863	.451
Hypoxia x Herbicide	15597.982	2	7798.991	.542	.598
Error	143921.746	10	14392.175		
Total	761287.356	16			
Corrected Total	226401.112	15			

a. R Squared = .364 (Adjusted R Squared = .046)

2. Growth parameters

2.1 Shoot length

Day 1:

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected model	2.556[a]	5	.511	2.000	.107
Intercept	277.778	1	277.778	1086.957	.000
Herbicide	.111	1	.111	.435	.515
Hypoxia	1.389	2	.694	2.717	.082
Hypoxia x Herbicide	1.056	2	.528	2.065	.144
Error	7.667	30	.256		
Total	288.000	36			
Corrected Total	10.222	35			

a. R Squared = .250 (Adjusted R Squared = .125)

Day 3:

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected model	2.153[a]	5	.431	.365	.868
Intercept	930.250	1	930.250	789.314	.000
Herbicide	.134	1	.134	.114	.738
Hypoxia	1.102	2	.551	.467	.631
Hypoxia x Herbicide	.917	2	.459	.389	.681
Error	35.357	30	1.179		
Total	967.760	36			
Corrected Total	37.510	35			

a. R Squared = .057 (Adjusted R Squared = -.100)

Day 7:

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected model	25.986[a]	5	5.197	2.207	.080
Intercept	1357.923	1	1357.923	576.517	.000
Herbicide	7.380	1	7.380	3.133	.087
Hypoxia	14.052	2	7.026	2.983	.066
Hypoxia x Herbicide	4.554	2	2.277	.967	.392
Error	70.662	30	2.355		
Total	1454.570	36			
Corrected Total	96.648	35			

a. R Squared = .269 (Adjusted R Squared = .147)

2.2 Root length

Day 1:

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected model	236.594[a]	5	47.319	2.073	.088
Intercept	12887.130	1	12887.130	564.455	.000
Herbicide	9.100	1	9.100	.399	.531
Hypoxia	225.762	2	112.881	4.944	.012
Hypoxia x Herbicide	1.732	2	.866	.038	.963
Error	958.906	42	22.831		
Total	14082.630	48			
Corrected Total	1195.500	47			

a. R Squared = .198 (Adjusted R Squared = .102)

Day 3:

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected model	453.478[a]	5	90.696	4.456	.004
Intercept	13716.314	1	13716.314	673.912	.000
Herbicide	138.847	1	138.847	6.822	.014
Hypoxia	251.644	2	125.822	6.182	.006
Hypoxia x Herbicide	62.987	2	31.494	1.547	.229
Error	610.598	30	20.353		
Total	14780.390	36			
Corrected Total	1064.076	35			

a. R Squared = .426 (Adjusted R Squared = .331)

Day 7:

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected model	1613.036[a]	5	322.607	10.701	.000
Intercept	15985.388	1	15985.388	530.232	.000
Herbicide	1334.684	1	1334.684	44.271	.000
Hypoxia	181.967	2	90.984	3.018	.064
Hypoxia x Herbicide	96.384	2	48.192	1.599	.219
Error	904.437	30	30.148		
Total	18502.860	36			
Corrected Total	2517.472	35			

a. R Squared = .641 (Adjusted R Squared = .581)

3. Total free amino acid content

Day 1:

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected model	85115.265[a]	5	17023.053	1.453	.281
Intercept	3390495.364	1	3390495.364	289.326	.000
Herbicide	49647.364	1	49647.364	4.237	.064
Hypoxia	16188.846	2	8094.423	.691	.522
Hypoxia x Herbicide	7041.667	2	3520.833	.300	.746
Error	128904.500	11	11718.591		
Total	5005233.000	17			
Corrected Total	214019.765	16			

a. R Squared = .398 (Adjusted R Squared = .124)

Day 3:

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected model	347328.750[a]	5	69465.750	3.694	.024
Intercept	4992102.033	1	4992102.033	265.502	.000
Herbicide	332870.380	1	332870.380	17.704	.001
Hypoxia	31125.969	2	15562.984	.828	.457
Hypoxia x Herbicide	19455.489	2	9727.744	.517	.607
Error	263235.250	14	18802.518		
Total	5550744.000	20			
Corrected Total	610564.000	19			

a. R Squared = .569 (Adjusted R Squared = .415)

Day 7:

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected model	233617.417[a]	5	46723.483	3.953	.019
Intercept	3371355.130	1	3371355.130	285.248	.000
Herbicide	196373.348	1	196373.348	16.615	.001
Hypoxia	33744.667	2	16872.333	1.428	.273
Hypoxia x Herbicide	4661.381	2	2330.690	.197	.823
Error	165466.333	14	11819.024		
Total	3964985.000	20			
Corrected Total	399083.750	19			

a. R Squared = .585 (Adjusted R Squared = .437)

4. Soluble protein content

Day 1:

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected model	835.895[a]	5	167.179	.783	.578
Intercept	9536.426	1	9536.426	44.674	.000
Hypoxia	40.609	2	20.305	.095	.910
Herbicide	324.509	1	324.509	1.520	.238
Hypoxia x Herbicide	412.113	2	206.057	.965	.405
Error	2988.543	14	213.467		
Total	13425.864	20			
Corrected Total	3824.438	19			

a. R Squared = .219 (Adjusted R Squared = -.061)

Day 3:

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected model	333.910[a]	5	66.782	.501	.770
Intercept	5862.606	1	5862.606	44.011	.000
Hypoxia	11.171	2	5.585	.042	.959
Herbicide	79.301	1	79.301	.595	.455
Hypoxia x Herbicide	192.927	2	96.464	.724	.505
Error	1598.493	12	133.208		
Total	8494.736	18			
Corrected Total	1932.403	17			

a. R Squared = .173 (Adjusted R Squared = -.172)

Day 7:

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected model	190.790[a]	5	38.158	.415	.829
Intercept	4437.326	1	4437.326	48.311	.000
Hypoxia	42.572	2	21.286	.232	.797
Herbicide	163.550	1	163.550	1.781	.209
Hypoxia x Herbicide	13.855	2	6.927	.075	.928
Error	1010.344	11	91.849		
Total	6239.562	17			
Corrected Total	1201.134	16			

a. R Squared = .159 (Adjusted R Squared = -.224)

5. Carbohydrate content

5.1 Fructose

Day 1:

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected model	2.451[a]	5	.490	3.020	.059
Intercept	7.284	1	7.284	44.875	.000
Herbicide	.905	1	.905	5.574	.038
Hypoxia	.452	2	.226	1.392	.289
Hypoxia x Herbicide	.547	2	.273	1.685	.230
Error	1.785	11	.162		
Total	12.934	17			
Corrected Total	4.236	16			

a. R Squared = .579 (Adjusted R Squared = .387)

Day 3:

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected model	.942[a]	5	.188	.878	.522
Intercept	16.763	1	16.763	78.196	.000
Herbicide	.314	1	.314	1.464	.248
Hypoxia	.059	2	.029	.137	.873
Hypoxia x Herbicide	.453	2	.227	1.058	.375
Error	2.787	13	.214		
Total	21.294	19			
Corrected Total	3.728	18			

a. R Squared = .253 (Adjusted R Squared = -.035)

Day 7:

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected model	1.092[a]	5	.218	.976	.471
Intercept	36.607	1	36.607	163.571	.000
Herbicide	.700	1	.700	3.130	.102
Hypoxia	.112	2	.056	.250	.782
Hypoxia x Herbicide	.279	2	.140	.624	.552
Error	2.686	12	.224		
Total	40.384	18			
Corrected Total	3.777	17			

a. R Squared = .289 (Adjusted R Squared = -.007)

5.2 Glucose

Day 1:

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected model	12.056[a]	5	2.411	17.448	.000
Intercept	56.923	1	56.923	411.915	.000
Herbicide	4.081	1	4.081	29.534	.001
Hypoxia	4.377	2	2.189	15.837	.002
Hypoxia x Herbicide	3.918	2	1.959	14.175	.002
Error	1.106	8	.138		
Total	65.176	14			
Corrected Total	13.161	13			

a. R Squared = .916 (Adjusted R Squared = .863)

Day 3:

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected model	2.275[a]	5	.455	.940	.480
Intercept	17.692	1	17.692	36.567	.000
Herbicide	.386	1	.386	.797	.384
Hypoxia	1.111	2	.556	1.148	.341
Hypoxia x Herbicide	.620	2	.310	.641	.539
Error	8.225	17	.484		
Total	28.077	23			
Corrected Total	10.500	22			

a. R Squared = .217 (Adjusted R Squared = -.014)

Day 7:

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected model	2.635[a]	5	.527	1.315	.304
Intercept	35.868	1	35.868	89.481	.000
Herbicide	.369	1	.369	.921	.351
Hypoxia	1.751	2	.876	2.184	.143
Hypoxia x Herbicide	.393	2	.197	.490	.621
Error	6.814	17	.401		
Total	44.874	23			
Corrected Total	9.449	22			

a. R Squared = .279 (Adjusted R Squared = .067)

5.3 Sucrose

Day 1:

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected model	3326.887[a]	5	665.377	1.626	.210
Intercept	16543.144	1	16543.144	40.419	.000
Herbicide	2743.078	1	2743.078	6.702	.020
Hypoxia	233.220	2	116.610	.285	.756
Hypoxia x Herbicide	414.614	2	207.307	.506	.612
Error	6548.720	16	409.295		
Total	25898.004	22			
Corrected Total	9875.607	21			

a. R Squared = .337 (Adjusted R Squared = .130)

Day 3:

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected model	4107.539[a]	5	821.508	28.151	.000
Intercept	6532.064	1	6532.064	223.840	.000
Herbicide	2936.550	1	2936.550	100.629	.000
Hypoxia	341.387	2	170.693	5.849	.013
Hypoxia x Herbicide	489.890	2	244.945	8.394	.004
Error	437.728	15	29.182		
Total	11172.595	21			
Corrected Total	4545.267	20			

a. R Squared = .904 (Adjusted R Squared = .872)

Day 7:

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected model	24018.287[a]	5	4803.657	25.119	.000
Intercept	37904.493	1	37904.493	198.209	.000
Herbicide	22796.272	1	22796.272	119.206	.000
Hypoxia	450.102	2	225.051	1.177	.337
Hypoxia x Herbicide	164.314	2	82.157	.430	.659
Error	2677.288	14	191.235		
Total	55660.757	20			
Corrected Total	26695.576	19			

a. R Squared = .900 (Adjusted R Squared = .864)

5.4 Total soluble sugars

Day 1:

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected model	8655.923[a]	5	1731.185	4.601	.010
Intercept	27819.523	1	27819.523	73.939	.000
Herbicide	6101.680	1	6101.680	16.217	.001
Hypoxia	607.610	2	303.805	.807	.464
Hypoxia x Herbicide	2280.517	2	1140.258	3.031	.078
Error	5643.738	15	376.249		
Total	39624.588	21			
Corrected Total	14299.660	20			

a. R Squared = .605 (Adjusted R Squared = .474)

Day 3:

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected model	4106.397[a]	5	821.279	23.577	.000
Intercept	7906.968	1	7906.968	226.992	.000
Herbicide	2846.479	1	2846.479	81.716	.000
Hypoxia	373.053	2	186.527	5.355	.018
Hypoxia x Herbicide	539.746	2	269.873	7.747	.005
Error	522.505	15	34.834		
Total	12687.151	21			
Corrected Total	4628.902	20			

a. R Squared = .887 (Adjusted R Squared = .849)

Day 7:

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected model	36740.411[a]	5	7348.082	11.101	.000
Intercept	61424.300	1	61424.300	92.796	.000
Herbicide	35625.297	1	35625.297	53.820	.000
Hypoxia	284.716	2	142.358	.215	.809
Hypoxia x Herbicide	367.717	2	183.859	.278	.761
Error	9928.933	15	661.929		
Total	103783.591	21			
Corrected Total	46669.343	20			

a. R Squared = .787 (Adjusted R Squared = .716)

5.5 Starch

Day 1:

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected model	3037.085[a]	5	607.417	2.945	.051
Intercept	2330.388	1	2330.388	11.300	.005
Herbicide	1136.091	1	1136.091	5.509	.034
Hypoxia	896.233	2	448.116	2.173	.151
Hypoxia x Herbicide	763.602	2	381.801	1.851	.193
Error	2887.183	14	206.227		
Total	8356.978	20			
Corrected Total	5924.268	19			

a. R Squared = .513 (Adjusted R Squared = .339)

Day 3:

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected model	880.129[a]	5	176.026	7.016	.001
Intercept	1136.734	1	1136.734	45.308	.000
Herbicide	855.696	1	855.696	34.107	.000
Hypoxia	.202	2	.101	.004	.996
Hypoxia x Herbicide	24.232	2	12.116	.483	.625
Error	451.600	18	25.089		
Total	2468.464	24			
Corrected Total	1331.730	23			

a. R Squared = .661 (Adjusted R Squared = .567)

Day 7:

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected model	1231.001[a]	5	246.200	3.338	.026
Intercept	1659.088	1	1659.088	22.492	.000
Herbicide	643.972	1	643.972	8.730	.008
Hypoxia	158.013	2	79.006	1.071	.364
Hypoxia x Herbicide	429.016	2	214.508	2.908	.080
Error	1327.762	18	73.765		
Total	4217.851	24			
Corrected Total	2558.763	23			

a. R Squared = .481 (Adjusted R Squared = .337)

B. APPENDIX - CHAPTER 3

Figure S3.1. The *in vitro* activities of pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) in the leaves (a and c) and the roots (b and d) of wild-type (wt) *Arabidopsis thaliana* Col-0, *aldh7b4 and 35S::ALDH7B4* mutant plants, untreated (control) or treated with imazamox or glyphosate for 3 days. Values represent the mean ± SE (n = 5 biological replicates). Significant variations are marked with ● for differences between control and imazamox-treated plants, and with ● for differences between control and glyphosate-treated plants (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).

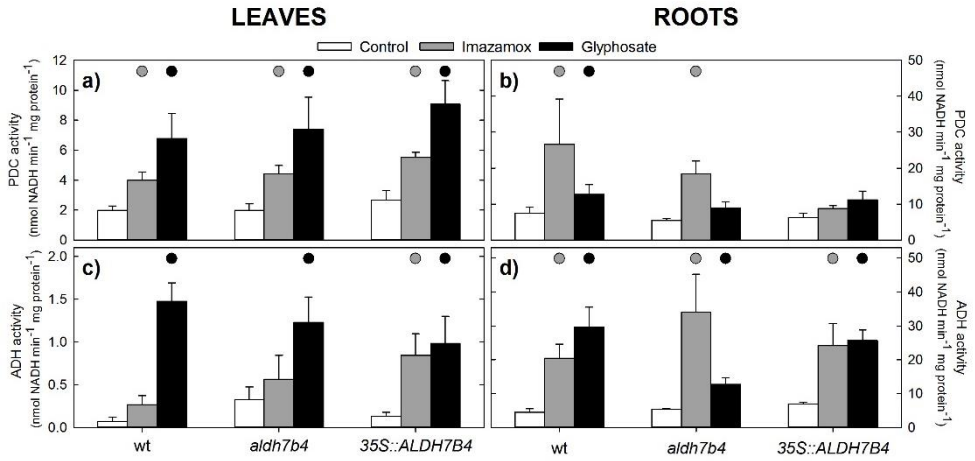


Figure S3.2. Overview of amino acid biosynthesis in plants (modified from Coruzzi and Last, 2000). Each group (A-H) is presented in the following figures with the corresponding colour.

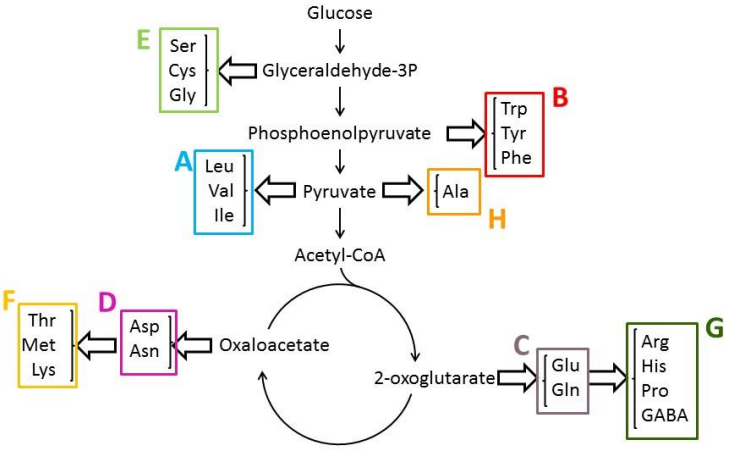


Figure S3.3. Total free amino acid content in the leaves (a) and the roots (b) of wild-type (wt) *Arabidopsis thaliana* Col-0, *aldh7b4 and 35S::ALDH7B4* mutant plants, untreated (control) or treated with imazamox or glyphosate for 3 days. Values represent the mean ± SE (n = 5 biological replicates). Significant variations are marked with ● for differences between control and imazamox-treated plants, and with ● for differences between control and glyphosate-treated plants (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).

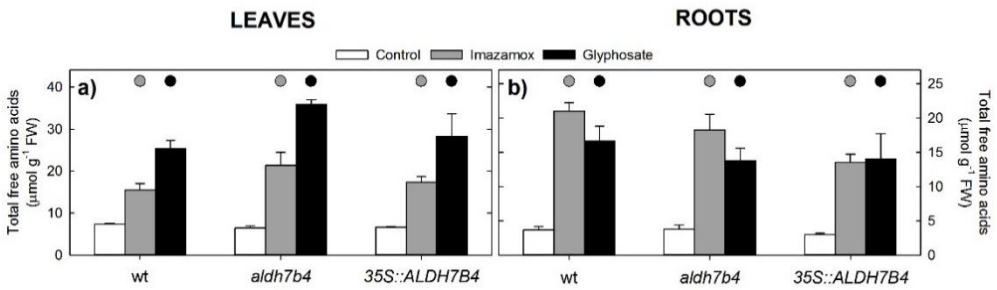
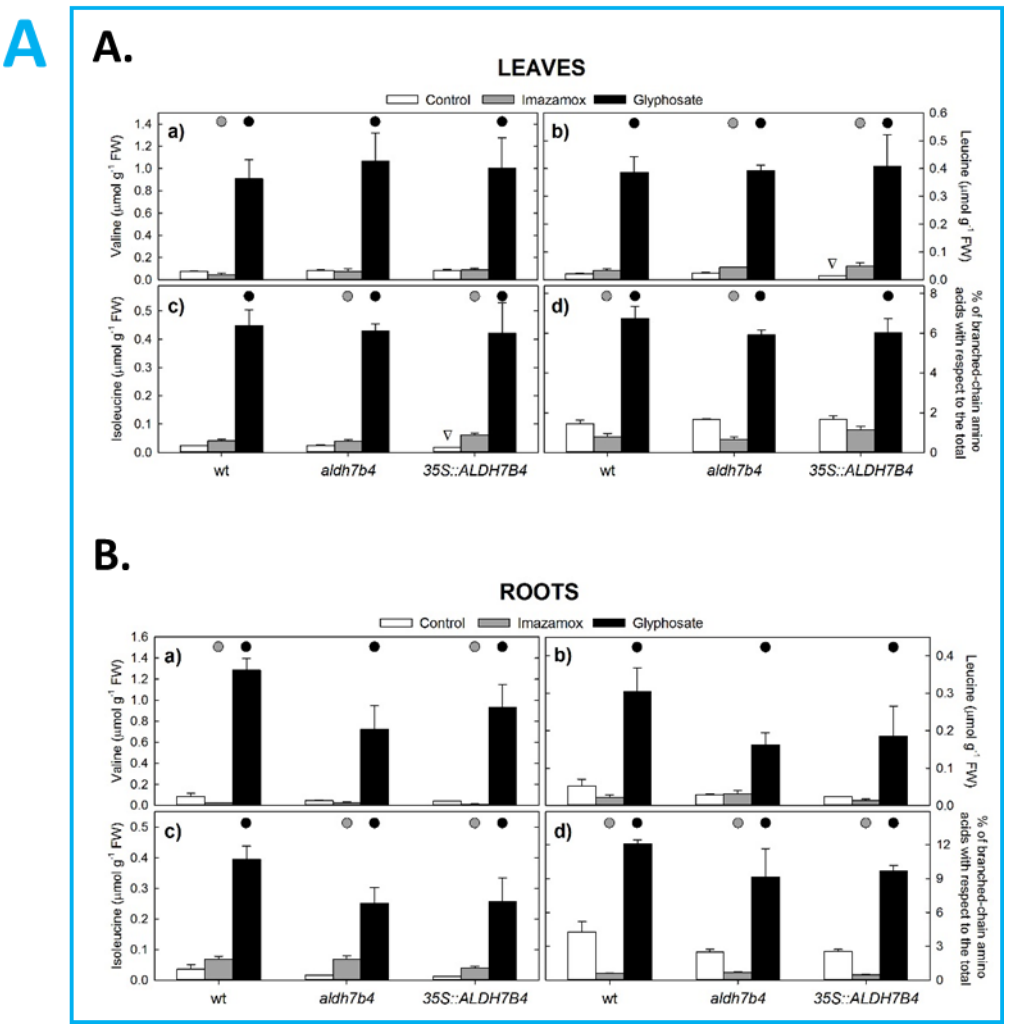


Figure S3.4. Branched-chain amino acid content in the leaves (A) and the roots (B) of wild-type (wt) *Arabidopsis thaliana* Col-0, *aldh7b4 and 35S::ALDH7B4* mutant plants, untreated (control) or treated with imazamox or glyphosate for 3 days. Values represent the mean ± SE (n = 5 biological replicates). ∇ indicates differences between the untreated plants of the corresponding genotype and the untreated wt plants (*t*-Test, $p < 0.05$). Significant variations are marked with ● for differences between control and imazamox-treated plants, and with ● for differences between control and glyphosate-treated plants (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).



FigureS3.5. Aromatic amino acid content in the leaves (A) and the roots (B) of wild-type (wt) *Arabidopsis thaliana* Col-0, *aldh7b4 and 35S::ALDH7B4* mutant plants, untreated (control) or treated with imazamox or glyphosate for 3 days. Values represent the mean ± SE (n = 5 biological replicates). Significant variations are marked with ● for differences between control and imazamox-treated plants, and with ● for differences between control and glyphosate-treated plants (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).

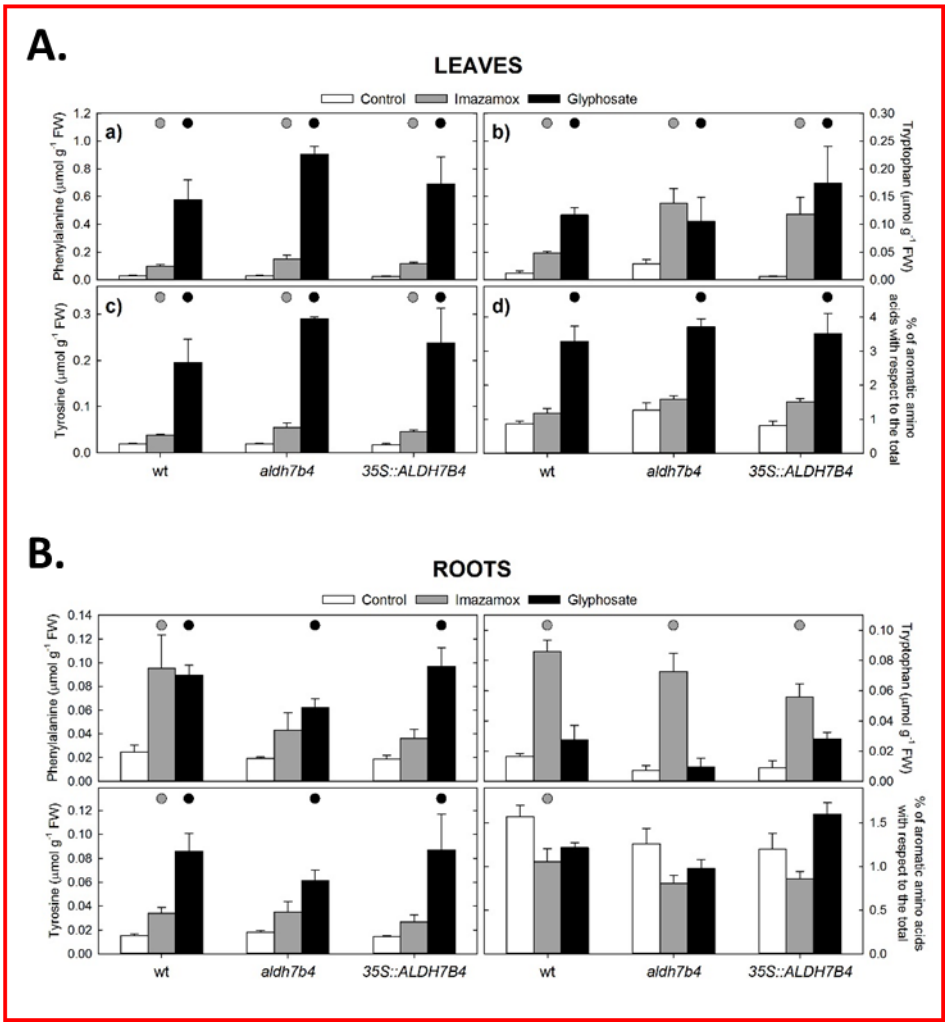


Figure S3.6. Acidic (glutamate and aspartate) and amide (glutamine and asparagine) amino acid content in the leaves of wild-type (wt) *Arabidopsis thaliana* Col-0, *aldh7b4 and 35S::ALDH7B4* mutant plants, untreated (control) or treated with imazamox or glyphosate for 3 days. Values represent the mean ± SE (n = 5 biological replicates). Significant variations are marked with ● for differences between control and imazamox-treated plants, and with ● for differences between control and glyphosate-treated plants (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).

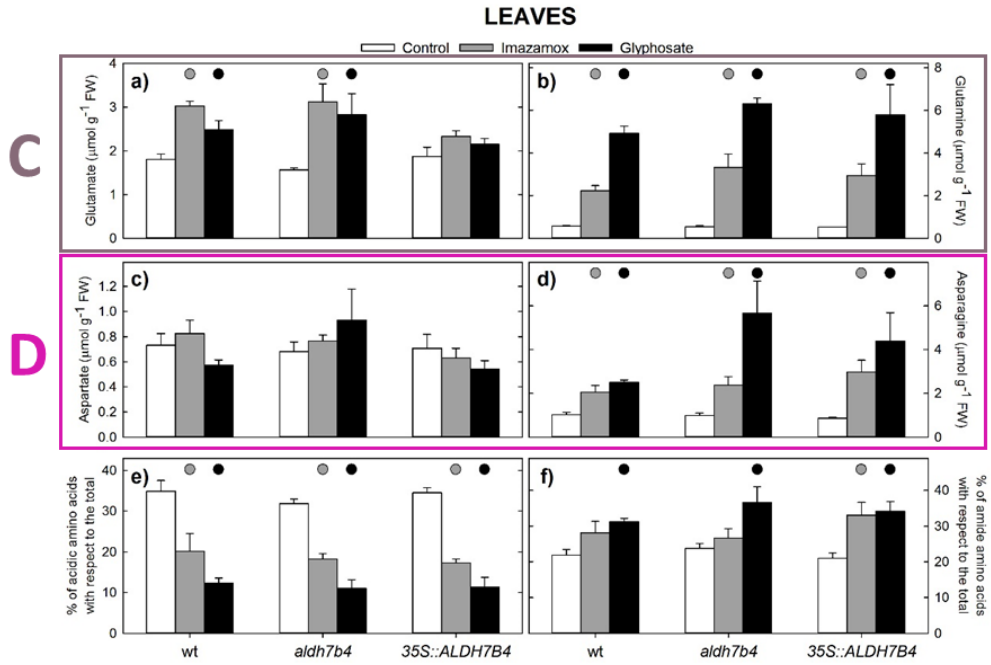


Figure S3.7. Acidic (glutamate and aspartate) and amide (glutamine and asparagine) amino acid content in the roots of wild-type (wt) *Arabidopsis thaliana* Col-0, *aldh7b4 and 35S::ALDH7B4* mutant plants, untreated (control) or treated with imazamox or glyphosate for 3 days. Values represent the mean ± SE (n = 5 biological replicates). ∇ indicates differences between the untreated plants of the corresponding genotype and the untreated wt plants (*t*-Test, $p < 0.05$). Significant variations are marked with ● for differences between control and imazamox-treated plants, and with ● for differences between control and glyphosate-treated plants (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).

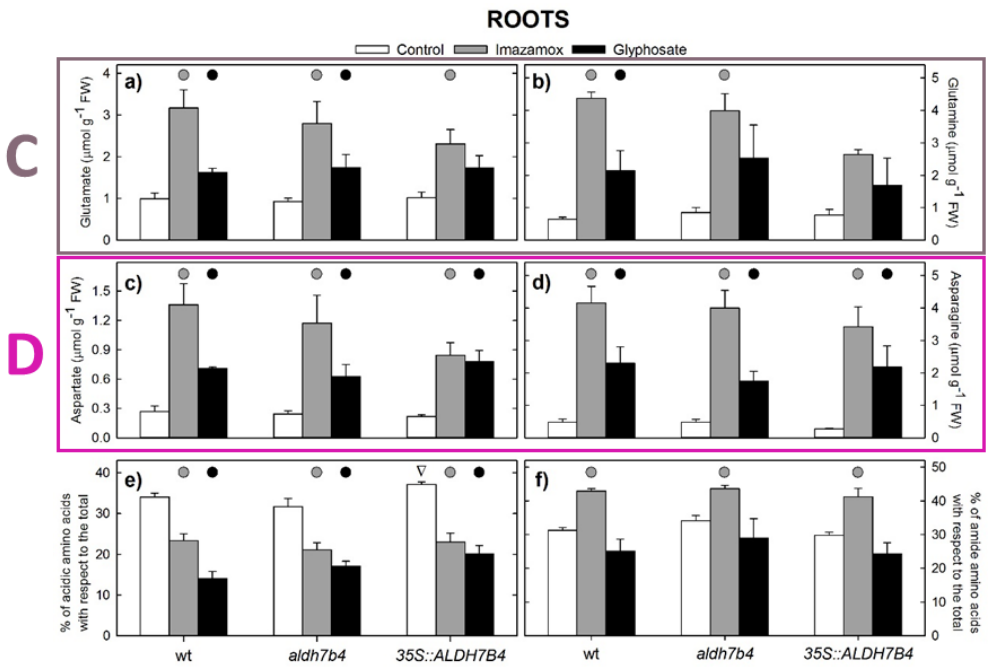


Figure S3.8. Serine (a), threonine (b), cysteine (c), methionine (d), glycine (e) and lysine (f) content in the leaves of wild-type (wt) *Arabidopsis thaliana* Col-0, *aldh7b4 and 35S::ALDH7B4* mutant plants, untreated (control) or treated with imazamox or glyphosate for 3 days. Values represent the mean ± SE (n = 5 biological replicates). Significant variations are marked with ● for differences between control and imazamox-treated plants, and with ● for differences between control and glyphosate-treated plants (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).

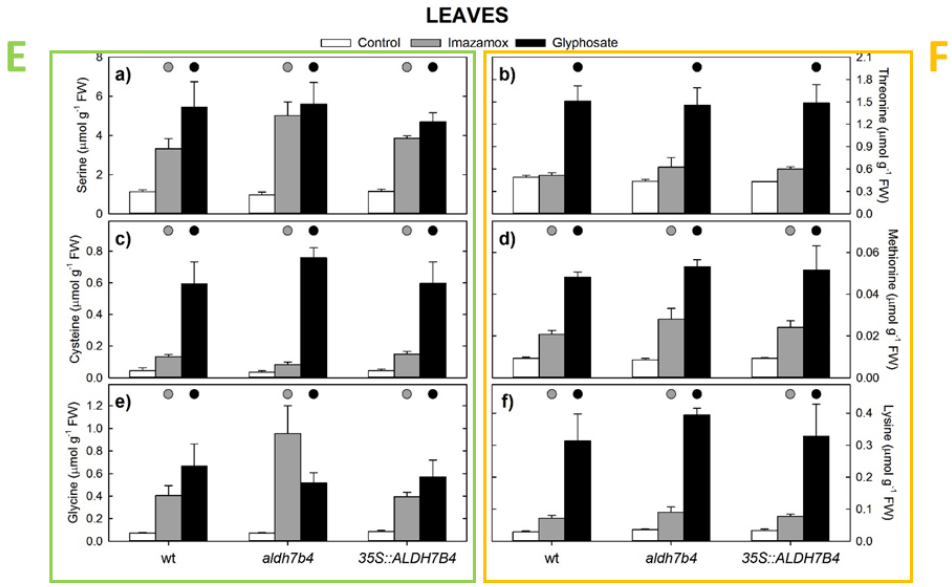


Figure S3.9. Serine (a), threonine (b), cysteine (c), methionine (d), glycine (e) and lysine (f) content in the roots of wild-type (wt) *Arabidopsis thaliana* Col-0, *aldh7b4 and 35S::ALDH7B4* mutant plants, untreated (control) or treated with imazamox or glyphosate for 3 days. Values represent the mean ± SE (n = 5 biological replicates). Significant variations are marked with ● for differences between control and imazamox-treated plants, and with ● for differences between control and glyphosate-treated plants (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).

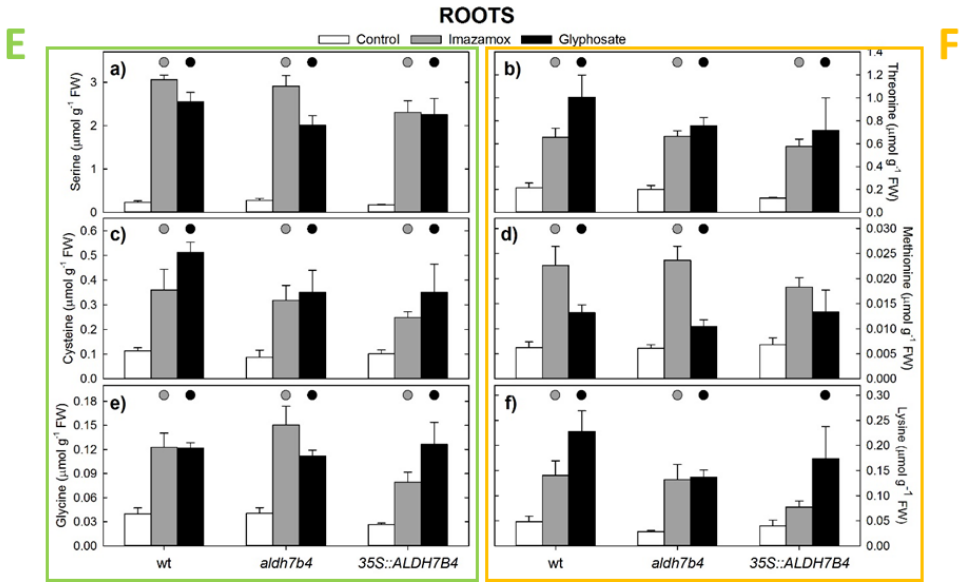


Figure S3.10. Arginine (a), histidine (b), proline (c), γ-aminobutyric acid (GABA) (d) and alanine (e) content in the leaves of wild-type (wt) *Arabidopsis thaliana* Col-0, *aldh7b4 and 35S::ALDH7B4* mutant plants, untreated (control) or treated with imazamox or glyphosate for 3 days. Values represent the mean ± SE (n = 5 biological replicates). Significant variations are marked with ○ for differences between control and imazamox-treated plants, and with ● for differences between control and glyphosate-treated plants (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).

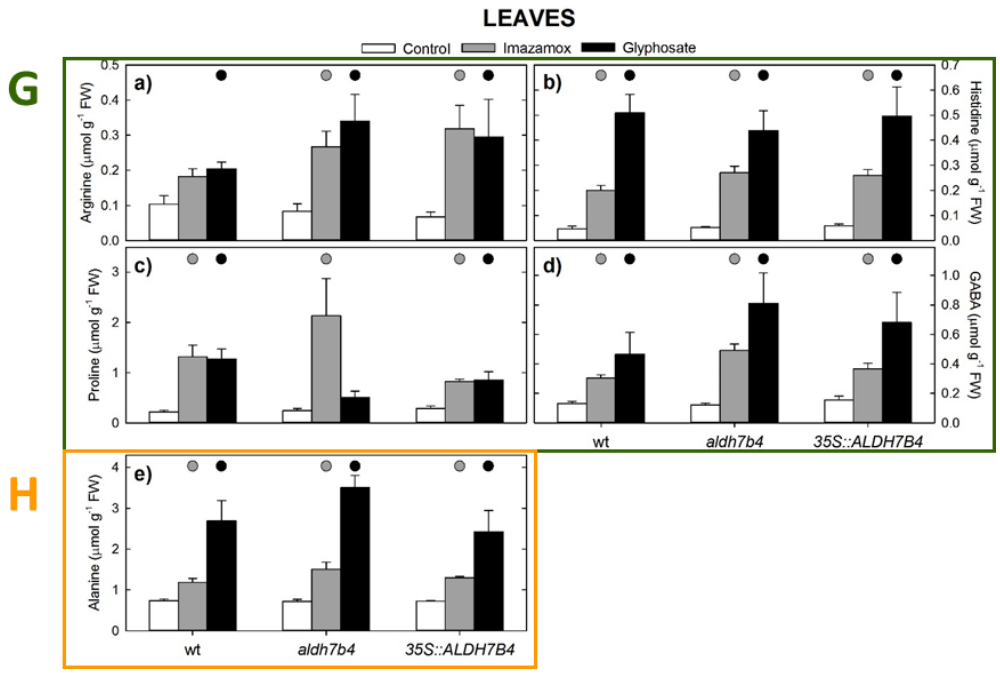


Figure S3.11. Arginine (a), histidine (b), proline (c), γ-aminobutyric acid (GABA) (d) and alanine (e) content in the roots of wild-type (wt) *Arabidopsis thaliana* Col-0, *aldh7b4 and 35S::ALDH7B4* mutant plants, untreated (control) or treated with imazamox or glyphosate for 3 days. Values represent the mean ± SE (n = 5 biological replicates). Significant variations are marked with ● for differences between control and imazamox-treated plants, and with ● for differences between control and glyphosate-treated plants (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).

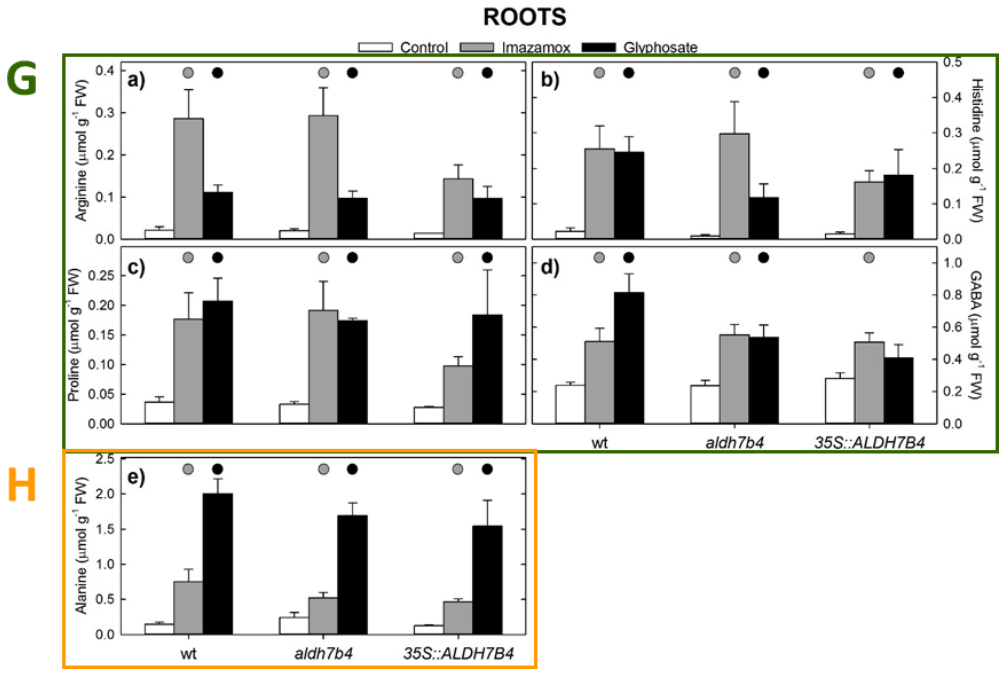


Figure S3.12. The carbohydrate content in the leaves of wild-type (wt) *Arabidopsis thaliana* Col-0, *aldh7b4 and 35S::ALDH7B4* mutant plants, untreated (control) or treated with imazamox or glyphosate for 3 days. Values represent the mean ± SE (n = 5 biological replicates). ▽ indicates differences between the untreated plants of the corresponding genotype and the untreated wt plants (*t*-Test, $p < 0.05$). Significant variations are marked with ● for differences between control and imazamox-treated plants, and with ● for differences between control and glyphosate-treated plants (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).

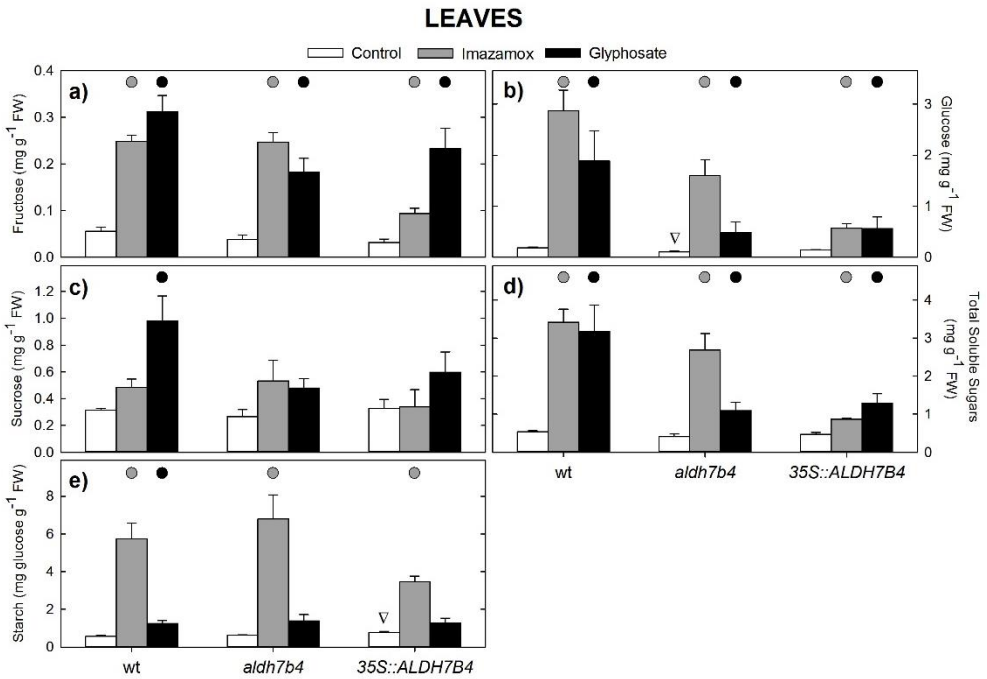


Figure S3.13. The carbohydrate content in the roots of wild-type (wt) *Arabidopsis thaliana* Col-0, *aldh7b4 and 35S::ALDH7B4* mutant plants, untreated (control) or treated with imazamox or glyphosate for 3 days. Values represent the mean ± SE (n = 5 biological replicates). ▽ indicates differences between the untreated plants of the corresponding genotype and the untreated wt plants (*t*-Test, $p < 0.05$). Significant variations are marked with ● for differences between control and imazamox-treated plants, and with ● for differences between control and glyphosate-treated plants (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).

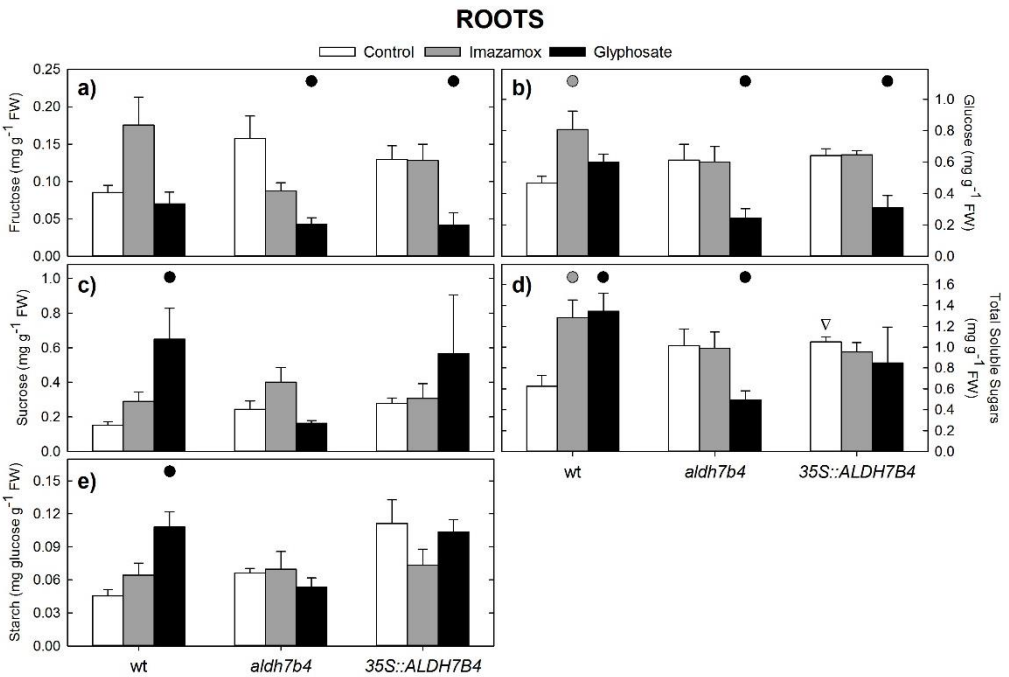


Figure S3.14. Organic acid content in the leaves of wild-type (wt) *Arabidopsis thaliana* Col-0, *aldh7b4 and 35S::ALDH7B4* mutant plants, untreated (control) or treated with imazamox or glyphosate for 3 days. Values represent the mean ± SE (n = 5 biological replicates). Significant variations are marked with ● for differences between control and imazamox-treated plants, and with ● for differences between control and glyphosate-treated plants (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).

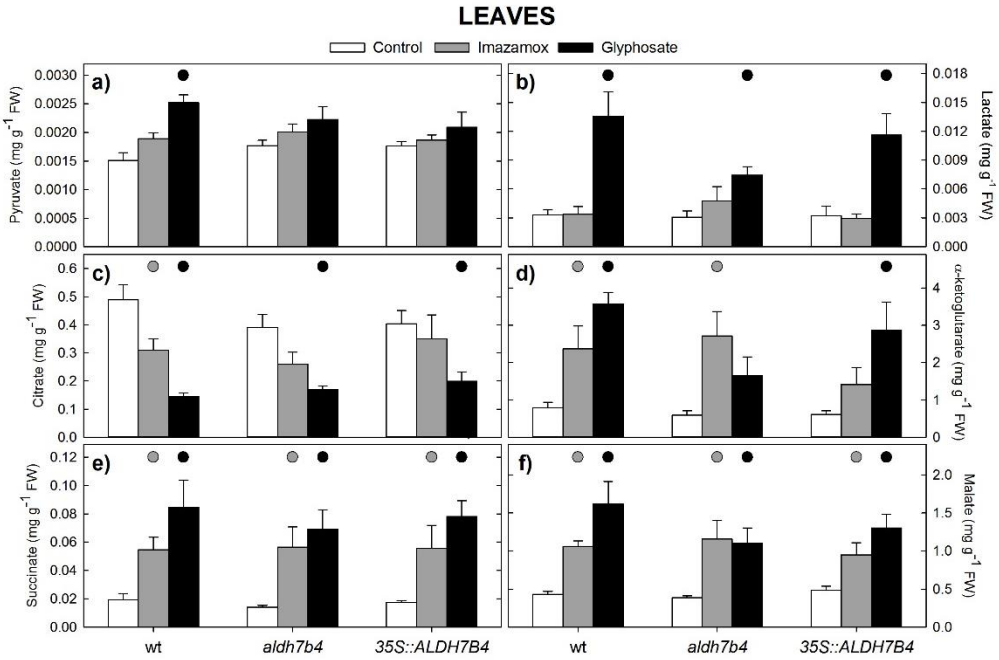


Figure S3.15. Organic acid content in the roots of wild-type (wt) *Arabidopsis thaliana* Col-0, *aldh7b4 and 35S::ALDH7B4* mutant plants, untreated (control) or treated with imazamox or glyphosate for 3 days. Values represent the mean ± SE (n = 5 biological replicates). ▽ indicates differences between the untreated plants of the corresponding genotype and the untreated wt plants (*t*-Test, $p < 0.05$). Significant variations are marked with ● for differences between control and imazamox-treated plants, and with ● for differences between control and glyphosate-treated plants (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).

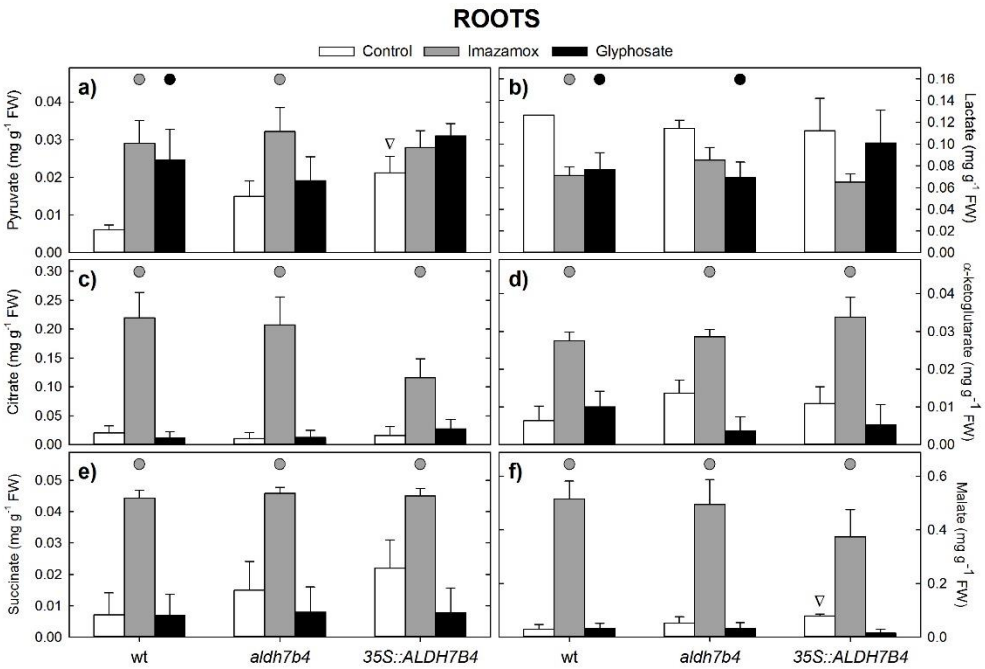


Figure S3.16. Shikimate (a and b) and quinate (c and d) content in the leaves and the roots of wild-type (wt) *Arabidopsis thaliana* Col-0, *aldh7b4 and 35S::ALDH7B4* mutant plants, untreated (control) or treated with imazamox or glyphosate for 3 days. Values represent the mean ± SE (n = 5 biological replicates). Significant variations are marked with ● for differences between control and imazamox-treated plants, and with ● for differences between control and glyphosate-treated plants (ANOVA, HSD Tukey/T3 Dunnet; $p < 0.05$).